STUDIES ON LOUPING-ILL VIRUS VACCINE

by

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DECLARATION

This is to certify that I, Barbara Ann Shaw, have carried out the investigations reported herewith and have composed this thesis.

May 1981.

ABBREVIATIONS

BHK-21	Baby hamster kidney cell line
HA	Haemagglutination
HAI	Haemagglutination-inhibition
pfu	Plaque forming unit
CF	Complement-fixation
LS	Lamb serum
FBS	Foetal bovine serum
NBCS	Newborn calf serum
ABS	Adult bovine serum
PBS	Phosphate buffered saline
TPB	Tryptose phosphate broth
TV	Trypsin/versene
DMSO	Dimethylsulphoxide
DEAE-D	Diethylaminoethyl dextran
PEG	Polyethylene glycol
s.c.	Sub-cutaneous
i.d.	Intra-dermal
i.p.	Intra-peritoneal
i.m.	Intra-muscular
rpm	Revolutions per minute
CPE	Cytopathic effect
OD	Optical density
p.i.	Post-infection
BAU	Virus propagated in serum free media, concentrated by ultrafiltration

Abbreviations cont'd.

BAP	Virus propagated in serum free media, concentrated by PEG precipitation
BAM	Virus propagated in serum free media, concentrated by methanol precipitation
LSU	Virus propagated in BHK virus growth media, concentrated by ultrafiltration
LSP	Virus propagated in BHK virus growth media, concentrated by PEG precipitation
LSM	Virus propagated in BHK virus growth media, concentrated by methanol precipitation
EBA	Serum-free BHK-21 media

ABSTRACT OF THESIS

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Degree Doctor of Philosophy *Date* May 1981

Title of Thesis Studies on Louping-ill Virus Vaccine

Studies were undertaken to define the optimum dosage regimen for commercial louping-ill virus vaccine, to investigate the susceptibility of vaccinated yet seronegative sheep to challenge with live louping-ill virus and to determine the storage characteristics of the vaccine at refrigerated and room temperatures.

Vaccination studies confirmed that two 1ml doses were required to provoke an adequate immune response in the majority of animals and maximum antibody titres were produced when an interval of two to eight weeks between injections was employed. Vaccinated animals were clinically resistant to experimental challenge although in animals exhibiting minimal or undetectable post-vaccinal antibody titres viraemias of low intensity were demonstrated. Circulating virus was not detected in animals with pre-challenge antibody titres greater than 1/10. Studies concerning vaccine stability demonstrated that both the virus antigen concentrate and the emulsified final vaccine were immunologically unstable following storage for periods of less than six months.

Production of louping-ill virus vaccine was therefore reappraised in an attempt to improve vaccine potency and stability and to produce a one-dose vaccine capable of consistently producing a satisfactory immune response in sheep.

Optimal conditions for the growth of louping-ill virus in BHK-21 cell monolayer cultures were determined experimentally and using this technique under commercial conditions titres of harvests ranged from 8.5 $\log_{10}$pfu/ml to 6.1 $\log_{10}$pfu/ml. A direct correlation between the preinactivation infectivity titres of virus suspensions and the immunogenicity of resulting vaccines was demonstrated and a threshold titre of 8.0 $\log_{10}$pfu/ml determined. Harvests with preinactivation infectivity titres below the threshold produced unacceptable vaccines. Concentration of virus antigen was therefore required to consistently produce potent louping-ill virus vaccine.

The present commercial vaccine is manufactured by concentrating inactivated virus harvests by methanol precipitation. A study of the efficacy of this concentration technique demonstrated however that there was no increase in the immunogenicity of concentrated virus harvests compared to unconcentrated virus harvests and in several instances concentration resulted in vaccines of decreased potency. The low potency characteristic of the commercial vaccine may therefore be partly attributable to the ineffectiveness of this technique for concentrating viral antigen.

Concentration by methods other than methanol precipitation were examined; these were ultrafiltration and precipitation with polyethylene glycol. Vaccines produced from virus antigen concentrated by ultrafiltration and prepared under both experimental and commercial conditions of production were consistently more immunogenic than corresponding vaccines prepared by concentrating viral antigen by methanol precipitation or polyethylene glycol precipitation. A single 1ml dose and two 1ml doses of vaccine prepared by ultrafiltration produced a satisfactory immune response in sheep and cattle respectively and antibody titres remained constant for at least six months.

Stability studies with vaccine prepared by ultrafiltration and stored at refrigerated temperatures demonstrated that although the immunogenicity of the emulsified final vaccine progressively declined over a six month period, immunogenicity of the virus antigen concentrate remained stable. Further work therefore is required to improve stability of the final vaccine and it is suggested that this be designed to evaluate the use of alternate adjuvants, preservative agents and storage conditions. Use this side only.

CHAPTER ONE

INTRODUCTION

Louping-ill is a tick transmitted encephalomyelitis primarily affecting sheep. The causative agent is recognised as a member of the group of closely related tick-borne flaviviruses belonging to the family of viruses known as the Togaviridae (Andrewes, Pereira and Wildy, 1978).

In the 19th and early 20th centuries the term 'louping-ill' was commonly used by shepherds in Scotland and parts of north England to describe a group of disease conditions in sheep that included pyaemic spinal meningitis, gastritis, woolball, joint-ill and pneumonia (Cleishbottam, 1816; Duncan, 1807; M'Fadyean, 1894, 1900). Pool, Brownlee and Wilson (1930), following a systematic investigation of louping-ill, described the first successful in series transmission of 'true' louping-ill in sheep by intracerebral inoculation with material from the central nervous system of an affected animal.

The common vector of louping-ill proved to be the tick Ixodes ricinus, trans-stadial but not transovarial transmission being demonstrated by MacLeod and Gordon (1932). Experimental transmission by certain non-British species of ticks has also been demonstrated and these include Rhipicephalus appendiculatus (Alexander and Neitz, 1933) and Hyalomma anatolicum (Swanepoel, 1968). I. ricinus is capable of feeding on any vertebrate species (Arthur, 1963) and there is evidence that several wild vertebrate species are naturally infected with louping-ill: shrew (Sorex craneus), wood mouse (Apodemus sylvaticus) (Smith, Varma and McMahon, 1964), red deer (Cervus elaphus) (Dunn, 1960; Adam, Beasley and Blewett, 1977),

red grouse (Lagopus lagopus scoticus) (Williams, Thorburn and Ziffo, 1963; Reid and Boyce, 1974), and roe deer (Capreolus capreolus) (Reid, Barlow, Boyce and Inglis, 1976).

Infection of the tick is effected by only those vertebrate species that develop viraemias in excess of the threshold titre required to establish virus in the tick (Beasley, Campbell and Reid, 1978). Following a study of the course of infection in a number of candidate hosts Reid (1978), suggested that only sheep were likely to develop viraemias of a sufficient intensity to regularly transmit virus to the tick, and concluded that sheep were probably primarily responsible for the maintenance of louping-ill in nature.

Although clinical disease is generally associated with sheep, naturally occurring disease in other domestic animals has also been described: cattle (Dunn, 1952), horses (Fletcher and Galloway, 1937; Timoney, 1980), pigs (Bannatyne, Wilson, Reid, Buxton and Pow, 1980) and dogs (MacKenzie, Smith and Muir, 1973). Man is also susceptible to louping-ill infection and as a result of occupational associations with the virus incidences of infection have been reported in laboratory workers (Rivers and Schwentker, 1933, 1934; Edward, 1948b, Webb, Connolly, Kane, O'Reilly and Simpson, 1968) and in butchers and abattoir workers (Williams and Thorburn, 1962; Lawson, Manderson and Hurst, 1949). The number of cases of natural infection in man which has been recorded has remained small (Davison, Neubauer and Hurst, 1948; Brewis Neubauer and Hurst, 1949; Likar and Dane, 1958; Ross, 1961).

Experimentally louping-ill infection has been studied in laboratory animals (Alston and Gibson, 1931; Czarkowska-Gladney and

Hurst, 1931; Hurst, 1931; Grešíková, Albrecht and Ernek, 1961; Webster and Fite, 1933; Doherty, 1969a, 1969b; Zlotnik, Carter and Grant, 1971), in domestic species (Pool, Brownlee and Wilson, 1930; Brownlee and Wilson, 1932; Dunn, 1952; Dow and McFerran, 1964; Reid and Doherty, 1971a, 1971b) and captive wild species (Findlay and Elton, 1933, Seamer and Zlotnik, 1970; Reid 1975b, 1978; Reid, Moss, Pow and Buxton, 1980).

Elford and Galloway (1933) using colloidal membranes estimated the size of louping-ill virus to be 15-20 millimicrons and Lépine (1931) demonstrated that the optimal pH for viral stability was 7.5 - 8.5. The virus is inactivated by treatment with formalin (Gordon, Brownlee, Wilson and MacLeod, 1932), bile salts (Smith, 1939), ethyl ether (Andrewes and Horstmann, 1949), heat (Lépine, 1931), saponin and sodium lauryl sulphate (Burnet and Lush, 1940).

The disease in sheep is biphasic. In the first febrile phase virus multiplies and can be readily detected in the blood of the animal. The second febrile phase follows a few days later coupled with, in a proportion of cases, incoordination, paralysis and death. Gordon, Brownlee, Wilson and MacLeod (1932) and Edward (1947a) demonstrated that experimental infection of animals by the intracerebral route results in death. Following subcutaneous injection and in the naturally occurring disease the outcome of infection is variable although viral invasion of the central nervous system has been shown to be an inevitable consequence following both intracerebral and peripheral inoculation (Zlotnik, Keppie and Grant, 1970; Doherty and Reid, 1971a). Reid and Doherty (1971b) demonstrated that following peripheral challenge the level of circulating virus

increases exponentially for two to three days and then declines rapidly. The decline and cessation of viraemia is associated with the appearance of humoral antibody. Antibody specific to louping-ill virus is also produced locally in the central nervous system and the rate of appearance parallels that of serum antibody (Reid, Doherty and Dawson, 1971). Reid and Doherty (1971b) therefore suggested that the variable outcome of infection following peripheral challenge is dependent on the magnitude and duration of viraemia and the rate at which the immune system is activated.

Symptoms of louping-ill in sheep are caused by damage to the Purkinje cells of the cerebellum and the motor nuclei, vestibular nuclei and the anterior horn cells of the spinal cord (Doherty and Reid, 1971a). The symptoms of louping-ill disease vary according to the host (Schwentker, Rivers and Finkelstein, 1933). In monkeys ataxia is predominant and is caused by widespread disintegration of the Purkinje cells, in mice, however, the outstanding symptoms are incoordination, weakness and paralysis caused by destruction of cells in the cerebrum and spinal cord.

On tick infected pastures where louping-ill is enzootic, heavy losses, as high as 60 per cent of the flock, may be experienced. Losses occur in spring and autumn coinciding with the periods of maximum tick activity. New stock and young sheep returning to tick infected pastures after overwintering on tick-free ground are particularly vulnerable (Pool, Brownlee and Wilson, 1930).

Three principal strategies have been attempted to combat louping-ill; these are treatment, serum prophylaxis and vaccination.

Laidlaw (1872) first described a treatment for affected animals recommending a glass of good whisky for ewes and complete immersion in cold water for afflicted lambs; the latter treatment was said to have been used with considerable success.

Prophylactic immunization of lambs using hyper-immune louping-ill antiserum obtained from either sheep or horses was described by Wilson and Gordon (1948). The duration of passive immunity conferred by the sheep antiserum was approximately four weeks and was longer than that resulting from the horse antiserum. As a result of the short duration of immunity the technique was limited in value although in controlled field experiments high-titred sheep antiserum was shown to effect an appreciable reduction in lamb mortality.

The first attempt at vaccination of susceptible animals was described by Gordon, Brownlee, Wilson and MacLeod (1932). They investigated the use of live virus based on the observation of Greig, Brownlee, Wilson and Gordon (1931) that sheep inoculated subcutaneously with live virus were subsequently immune, but following field trials it was concluded that the method was particularly hazardous.

Interest then focused on the production of an inactivated virus vaccine using methods similar to those of Laidlaw and Dunkin (1928) for dog distemper vaccine. In 1931, a vaccine consisting of a formalinized 10 per cent suspension of brain, spinal cord and spleen tissues taken from experimentally infected sheep was subjected to field trials to assess immunological efficiency and innocuity (Gordon, 1934). Controlled large-scale field trials from 1931-1934 showed that an average mortality of 9 per cent in unvaccinated animals was reduced to less than one per cent in vaccinated animals

(Gordon, 1946). A vaccine dose schedule of two injections was required to produce detectable levels of neutralizing antibody and to protect sheep against challenge with live virus (Wilson and Gordon, 1948; Edward, 1947b).

The vaccine was issued commercially in 1935 as three separate batches and subsequently over 20,000 doses per annum were distributed. A potential hazard in this technique of vaccine production, i.e. the presence of adventitious agents in sheep tissue, became a reality in 1937 when a proportion of Blackface sheep inoculated in 1935 with Batch 2 vaccine developed scrapie, a disease hitherto unknown in this breed. On investigation it was found that eight Cheviot lambs, born from ewes that had been in contact with scrapie infection had been included in the 114 sheep that had been used to make this batch of vaccine and it was presumed that although the lambs were apparently healthy they had been in the incubative stage of a scrapie infection (Gordon, 1946).

The vaccine was used without further incident until 1968 when it became apparent that the manufacturing methods involved constituted a serious hazard to production staff, and the vaccine was withdrawn from the market.

Edward (1947b, 1948a) developed louping-ill vaccines from infected chick embryo and infected mouse brain. Both vaccines were shown to protect sheep against challenge with live virus but the chick embryo vaccine proved to be unstable and lost immunogenicity following 17 weeks of storage. The mouse brain vaccine, although never considered for commercial use, was used over a period of 10 years to protect at risk personnel involved in the production of commercial louping-ill vaccine.

Wilson (1945) and Edward (1947a) attempted to attenuate the virus by repeated passage in cultured minced chick embryo and chick embryo respectively. Attempts were however unsuccessful as virus at the 64th pass in chick embryo and the 170th pass in crude tissue culture was still fully virulent for mice and sheep.

O'Reilly, Smith, McMahon, Wilson and Robertson (1965) investigated the use of Langat virus strain TP-21 as a live vaccine for the protection of sheep against louping-ill. The antibody responses following vaccination were however low and its use as a vaccine was not recommended.

Mayer, Blaškovič, Ernek and Libíková (1969) studying the immunogenic and antigenic properties of a monkey and mouse attenuated clone, designated Hy-Hk 28 '2' of the tick-borne encephalitis (Western subtype) virus demonstrated that vaccination of sheep with a single dose of this virus produced seroconversion in 85 per cent of animals and that 11 months after vaccination animals were protected against challenge with live louping-ill virus.

An inactivated vaccine based on louping-ill virus propagated in secondary sheep kidney cell cultures was developed by Brotherston and Boyce (1970); the infected sheep kidney tissue culture fluids were treated with formalin and precipitated with cold methanol. The precipitate was resuspended in saline and mixed with oil adjuvant to form the finished vaccine. A single dose of this vaccine was shown to elicit the production of haemagglutination inhibiting antibodies and neutralizing antibodies and to protect sheep against challenge with live virus (Brotherston, Bannatyne, Mathieson and Nicholson, 1971).

This method of vaccine production was offered for commercial exploitation and in 1971 a vaccine based on this technique of preparation but with the exception that the virus was propagated in BHK-21 cells was issued commercially. The current average annual sales of the vaccine are in the region of 200,000 doses. The vaccine was originally issued as a single dose vaccine but experience indicated that the potency of this vaccine was considerably lower than that of the experimental vaccine described by Brotherston and Boyce (1970). Two doses of commercial vaccine are now required to consistently provoke a satisfactory immune response. It has however been demonstrated that even following two doses of vaccine a proportion of sheep remain seronegative. In addition regular potency testing of the commercial vaccine indicated that the vaccine potency progressively diminished as the storage period of the vaccine increased. The stability characteristics of commercial vaccine at refrigerated temperatures are undetermined.

A requirement therefore exists to define the time interval between doses giving the maximum serological response, to determine the relationship between antibody response and the protection afforded and to determine the storage characteristics of the vaccine.

Louping-ill is probably the most important virus disease of sheep affecting the hill farming areas of Scotland and northern England. A double dose vaccination schedule, however effective at protecting sheep from infection, is not readily accommodated within the practical management policies of the hill farms. In addition as a result of the two dose requirement vaccine production and purchase costs are increased. An effective single dose vaccine would therefore be considerably more attractive to both hill farmer and vaccine

manufacturer.

In addition therefore a reappraisal of the present louping-ill vaccine production procedure is also required in an attempt to develop a vaccine capable of consistently provoking a satisfactory serological response following a single dose.

The studies reported here were therefore undertaken with the aim of defining the optimum dosage requirements and the stability of the presently available vaccine, to determine the relationship between antibody response and protection and to develop a single dose vaccine of increased potency and stability.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

1. CELLS

The stable cell line BHK-21 (clone 13) (MacPherson and Stoker, 1962) was obtained from the Wellcome Research Laboratories, Beckenham, at the 77th passage. All experimental work was performed using cells between the 92nd and 112th pass levels. This was carried out by preparing three tissue culture flasks* from cells at the 89th pass. After 72 hours incubation, when a confluent monolayer had formed, the BHK cell growth medium was decanted and the cells washed with phosphate buffered saline (PBS) pH 7.2 without calcium or magnesium (Dulbecco and Vogt, 1954). The cells were detached using 10ml of a 0.1 per cent (w/v) trypsin - 0.05 per cent (w/v) versene solution. The resulting cell suspension was centrifuged at 700g for 20 minutes and the supernatant removed. BHK cell growth media containing 20 per cent newborn calf serum (NBCS) and 10 per cent dimethyl sulphoxide (DMSO) was then added to give a cell concentration of 5×10^6 cells/ml. This cell suspension was then dispensed in 3ml volumes in screw-capped bottles, placed in a polystyrene container and stored at $-80^{\circ}C$. After 24 hours the bottles were transferred for storage in liquid nitrogen. Cells were removed at intervals and cultures prepared to maintain a supply of cells at the required pass level.

Cells were grown in tissue culture flasks and passaged every 3-4 days at an expansion rate of 1:3. The medium was decanted and

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*Flow Laboratories, Irvine.

the cells washed and detached as described previously. The cells from each flask were then redistributed in a further three bottles in fresh cell growth media.

The BHK-21 (clone 13) R8 mycoplasma-free cell line, BHK-21 (R8MF) was obtained from the Wellcome Foundation, Pirbright, at the 118th passage. All experimental work was performed using cells between the 123rd and 126th pass levels. This was achieved by preparing a stock of cells at the 121st passage and treating them as described above using BHK cell growth media supplemented with a further 5 per cent (v/v) NBCS and 0.1 per cent (w/v) sodium bicarbonate.

The BHK-21 (R8MF) cells were periodically checked for the presence of mycoplasmas; this was kindly performed by Dr. G. E. Jones, Moredun Research Institute, Edinburgh.

The IB/RS2 pig kidney (PK) cell line was obtained from the Animal Virus Research Institute, Pirbright. Adsorption of virus to PK cells was achieved using cells between the 30th and 40th passage at the Moredun Research Institute.

Cells were cultured in PK cell growth media and passaged every 3-4 days at an expansion rate of 1:4.

2. MEDIA

BHK cell growth and virus growth media

	Cell growth	Virus growth
Eagles BHK 10x concentrate[1]	10% v/v	10% v/v
Tryptose phosphate broth	10% v/v	10% v/v
Newborn calf serum*	5% v/v	-
Lamb serum*	-	10% v/v
Sodium bicarbonate	0.1% w/v	0.2% w/v
Penicillin	200 i.u./ml	200 i.u./ml
Streptomycin	100 µg/ml	100 µg/ml

Reagents were obtained, with the exception of the serum, as stock solutions from the Wellcome Research Laboratories, Beckenham.

BHK serum free growth media (EBA)

Basic salt solution[2]	Earles
Bovine serum albumin[3]	0.5% w/v
Glucose	1% w/v
Sodium bicarbonate	0.1% w/v
Penicillin[4]	200 i.u./ml
Streptomycin[4]	100 i.u./ml

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[1] based on the original MacPherson and Stoker 1962 publication.

[2] without phenol red

[3] Miles Laboratories, Slough.

[4] BDH, Poole.

*mycoplasma screened

PK cell growth and virus growth media

	Cell growth	Virus growth
Basic salt solution	Hanks	Earles
Lactalbumin hydrolysate[1]	0.5% w/v	0.5% w/v
Adult bovine serum (inactivated)	10% v/v	10% v/v
Glucose	0.1% w/v	0.1% w/v
Sodium bicarbonate	0.02% w/v	0.2% w/v
Yeast Extract[1]	0.001% w/v	0.01% w/v
Folic acid	-	0.0001% w/v
Penicillin	200 i.u./ml	400 i.u./ml
Streptomycin	100 µg/ml	200 µg/ml
Polymyxin[2]	50 i.u./ml	-
Mycostatin[3]	50 i.u./ml	-

PKBA media

As for PK virus growth media substituting 0.5 per cent (w/v) bovine serum albumin for the 10 per cent adult bovine serum.

3. VIRUS

The standard Moredun isolate of virus Li/31 was employed for all laboratory procedures. The virus had been passaged intracerebrally in mice five times and once in BHK-21 cells. Virus stocks for all experiments were prepared by adsorbing 40ml of a 2 x 10^7 cells/ml BHK-21 cell suspension at the 89th pass level with 20ml of virus isolate Li/31.

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[1] Difco Laboratories Ltd., Detroit, Michigan, U.S.A.

[2] Wellcome Foundation Ltd., London,

[3] E.R. Squibb & Sons, Liverpool.

Adsorption proceeded at 37°C for 60 minutes. Four Wheaton* roller culture bottles were then each inoculated with 500ml of BHK virus growth media and 15ml of the virus/cell suspension and incubated at 37°C on a roller apparatus rotating at 0.3 rpm. After three days the infected fluids were decanted, pooled and centrifuged at 700g for 10 minutes at 4°C. The supernatant was distributed in 20ml volumes in screw-capped bottles, designated Li/31/M5 BHK/2, and stored at -80°C.

The Li/31 isolate at the 6th pass in mouse brain was used as reference antigen in immunodiffusion tests. This was prepared by injecting 10 litters of one-day-old mice with 0.03ml of a 10^{-3} dilution of clarified mouse brain infected with Li/31/M5. A 26-gauge needle was used for inoculation and 0.01ml was injected into the cranium and 0.02ml deposited subcutaneously (s.c.). The brains of the mice were harvested after five days and homogenized. A 10^{-1} dilution of this preparation was then sonicated for 30 seconds using a MSE 150 Watt Ultrasonic Disintegrator and then clarified at 10,000g for 30 minutes at 4°C. Normal mouse brains were homogenized, diluted and sonicated in the same way.

The SB526 virus isolate at the 2nd pass in sucking mouse brain (Reid and Doherty, 1971a) was used as inoculum in the challenge experiment.

4. VIRUS, VIRUS ANTIGEN AND ANTIBODY QUANTITATION.

Assay of virus infectivity

Virus infectivity was determined by the plaque assay method described by Reid and Doherty (1971b), using preformed monolayers of

*Jencons (Scientific) Ltd., Hemel Hempstead.

the IB/RS2 pig kidney cell line. Undiluted and diluted preparations of samples were inoculated onto four plates and results expressed as mean plaque forming units (pfu) per ml.

Immunofluorescence

The proportion of cells infected with virus following adsorption was determined by the direct immunofluorescence technique.

Preparation of fluorescein isothiocyanate (FITC) conjugated sheep anti-louping-ill globulin.

A hyperimmune serum sample was produced according to the method described by Doherty and Reid (1971b). A 5ml sample was dialysed against 0.01M phosphate 0.03M sodium chloride buffer pH 7.6 and the resultant serum applied to a Whatman DE/52 anion exchange column (15 x 20cm) equilibrated with the same buffer. The fall-through peak was pooled and concentrated by pressure dialysis against 0.05M carbonate buffer pH 9 and the final protein concentration adjusted to 20mg/ml. Celite with 10 per cent adsorbed FITC was added (2.5gm per ml of IgG), stirred for 5 minutes at room temperature and centrifuged at 1800g for 5 minutes at 4^{o}C. The supernatant was then applied to a Sephadex G25 column (1.5 x 10cm) equilibrated with IxPBS. The first peak containing conjugated IgG was collected.

Optimal conditions for direct immunofluorescence were obtained using the FITC sheep IgG conjugate at a dilution of 1/100. Pyrex glass tubes containing coverslips were inoculated with 1.5×10^{6} virus infected BHK-21 cells and incubated stationary at 37^{o}C. At intervals thereafter coverslips were removed, washed 3 times in PBS and fixed in cold acetone for 5 minutes. They were overlaid with a 1/100 dilution of the FITC sheep IgG conjugate and incubated in a

humid atmosphere at 37°C for one hour. The coverslips were then washed twice in PBS, dried and mounted in buffered glycerol pH 8.4. Examination for fluorescence was carried out using a Leitz* Ortholux 11 microscope fitted with a fluorescence vertical illuminator, the ultra-violet blue light being produced by a 50W high pressure mercury lamp.

Enumeration of infected cells was achieved by counting the number of fluorescent cells in 20 fields selected at random from appropriate coverslip preparations. The coverslips were then washed with water to remove the mounting fluid and re-examined under the same microscope readjusted to white light microscopy. Five fields with 50 per cent cell cover were chosen and the number of cells counted, then 20 fields were randomly selected and scanned for per cent cover. The percentage of infected cells was thus determined. Control uninfected coverslips were treated likewise.

Assay of haemagglutination (HA) activity

Haemagglutination activity in tissue culture harvests was assayed using the techniques described by Clarke and Casals (1958).

Samples (0.4ml volumes) were prepared by extracting with 20 volumes of chilled acetone for 5 minutes. The samples were then centrifuged at 1000g for 15 minutes at 4°C, the supernatant removed and the precipitate re-extracted with 20 volumes of a chilled mixture of equal parts acetone and ether. Following centrifugation the precipitate was dried on a vacuum air pump and resuspended in 0.4 per cent (w/v) bovalbumin borate saline (ph 9.0). The test was performed using goose erythrocytes and a final pH of 6.2 - 6.4 in standard leucite WHO plates.

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*Ernst Leitz (Canada) Ltd., Midland, Ontario.

Immunodiffusion

Concentrated virus antigen preparations were examined by immunodiffusion using double and radial diffusion methods.

For the double diffusion technique gels were prepared by pouring 15ml of one per cent Oxoid* Ionagar in PBS onto glass slides (8.2 x 8.2cm). Six circular wells, 5mm in diameter, were cut circumferentially around one well of the same diameter. All the wells were spaced 5mm apart. A 1/8 dilution of a sheep hyperimmune louping-ill antiserum, prepared according to the method described by Doherty and Reid (1971b) was used as antibody and a clarified 1/10 dilution of Li/31/M6 infected sucking mouse brain homogenate used as reference antigen. Normal mouse brain homogenate, BHK virus growth media and BHK cellular extracts concentrated 10 fold were also reacted against this antiserum. The concentrated BHK cellular extracts were prepared by freezing and thawing uninfected 72 hour monolayer cultures of BHK-21 cells, and concentrated by each of the concentration methods described.

The charged plates were incubated in a humid atmosphere at room temperature for 48 hours, then soaked in saline for 24 hours and distilled water for a further 24 hours. They were then dried, fixed with 2 per cent acetic acid for 5 minutes and stained with 0.5 per cent amido-schwartz 10B in a methanol (9 parts)-glacial acetic acid (1 part) mixture.

For the single radial diffusion technique antibody was incorporated into the agar at a final dilution of 1/100 and poured

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*Oxoid Ltd.

onto microscope slides (7.5 x 2.5cm). Four circular wells (2mm diameter) spaced 1.5cm apart were cut. The charged plates were incubated and stained as described previously.

Assay of haemagglutination-inhibiting (HAI) antibody.

Sera were pre-treated with kaolin to remove non-specific inhibitors of haemagglutination and tested for HAI activity using the method of Clarke and Casals (1958). The test was carried out in standard leucite WHO plates using 4-8 haemagglutination units of an acetone-ether extracted tissue culture antigen. To assess the relative activity of the IgM and IgG antibody class, positive sera were also treated at 64.5^{o}C for 30 minutes (Reid and Doherty, 1971a) and then examined for HAI activity.

CHAPTER THREE

THE IMMUNIZATION OF SHEEP WITH COMMERCIAL LOUPING-ILL VIRUS VACCINE; DEVELOPMENT OF ANTIBODY AND PROTECTION AGAINST CHALLENGE WITH LIVE VIRUS

INTRODUCTION

Experience with the commercially produced louping-ill-virus vaccine has indicated that a single dose of vaccine is often insufficient to provoke a satisfactory serological response. Preparation and potency testing of the commercial vaccine is described in Appendix 3.1.

This chapter describes the immune responses in sheep following the administration of a second injection of commercial vaccine after different time intervals and the effect of varying the dose volume. The response to viral challenge of those sheep which produced little or no antibody following vaccination is also examined.

MATERIALS AND METHODS

VACCINATION

Administration of a second dose of vaccine after different time intervals

Ninety, 9 month old Scottish Blackface HAI-negative sheep, bred and maintained in an area free of ticks were randomized into 9 equal groups. Groups I - VIII were injected subcutaneously (s.c.) behind the right shoulder with 1ml of commercial vaccine. Groups II - VIII were then revaccinated with a further 1ml of vaccine after one, 2, 3, 4, 8, 16 and 24 weeks respectively. Sheep in group IX were unvaccinated controls. Ten ml of blood were collected weekly for 4 weeks from all

animals following primary vaccination. Groups I and IX were then bled monthly for 7 months. Groups II to VIII were bled immediately prior to revaccination, then weekly for 4 weeks and monthly until completion of the experiment. The bloods were allowed to clot, the sera removed and stored at -20°C until tested for HAI activity using the method described in Chapter Two. A titre of <10 was considered a negative serological response.

For statistical analysis of the data reciprocal HAI titres were transformed into logarithms to the base 10. Titres of <10 were deemed to have a logarithmic value of 0.7. Group I - VII means were compared using an analysis of variance. Group VIII means which were excluded from this analysis because the data were significantly less variable, were compared with data from each of the other groups inturn using a 2-sample t-test and halving the number of degrees of freedom when required to allow for variance differences.

Administration of different dosage volumes

Twenty-one 9-month-old Cheviot HAI-negative sheep and 3 8-month-old Finnish Landrace HAI-negative sheep bred and maintained in an area free of ticks were randomized into 4 equal groups. They were injected subcutaneously behind the right shoulder with varying volumes of commercial vaccine and then revaccinated 21 days later. Groups A, C and D were vaccinated with 2 doses of 1ml, 2 doses of 0.5ml and 2 doses of 0.25ml respectively whilst group B were injected primarily with 1ml and then revaccinated with a further 0.5ml of vaccine. Blood was collected from all animals at weekly intervals for a period of 7 weeks and the sera tested for HAI activity using the method described in Chapter Two. The mean 21-day HAI titres and the mean 49-day HAI titres from each of the 4 groups were compared using an analysis of variance.

CHALLENGE OF VACCINATED SHEEP WITH LIVE VIRUS

In a group of vaccinated sheep a proportion of animals usually remain completely seronegative or fail to produce a persistent antibody response even after administration of 2 doses of vaccine (personal observation). In an attempt to assess the degree of protection afforded to such animals 26 of the 90 vaccinated sheep were challenged with live virus. Ten of the 26 sheep were the unvaccinated control sheep of group IX. The SB526 isolate of louping-ill virus was used as inoculum. The clarified mouse brain homogenate was diluted and sheep inoculated subcutaneously into the medial aspect of the right thigh with 1ml of inoculum containing 6.0 $\log_{10}$ pfu of virus. The sheep were bled immediately prior to challenge and then daily for 14 days. On each occasion two tubes of blood were collected; one was allowed to clot for serum separation whilst the other contained heparin.* The heparinised bloods were centrifuged at 1500g for 20 minutes at 4^{o}C, the supernatants removed and stored at -80^{o}C until assayed for virus using the plaque assay method as described in Chapter Two. The sera were stored at -20^{o}C until assayed for HAI activity.

The animals were inspected every 12 hours for clinical signs and, from those that died pieces of cerebrum, cerebellum and spinal cord were collected and stored at -80^{o}C until assayed for virus using the plaque method.

RESULTS

VACCINATION

Administration of a second dose of vaccine after different time intervals

The mean HAI titres of groups I - VIII 7 days after primary vaccination and the mean titres of groups II - VIII immediately prior

*Pullarin Evans Medical Ltd., Liverpool.

to revaccination are shown in Table 3.1. The numbers of animals responding to primary vaccination are also indicated. Immediately prior to administration of the second dose of vaccine a serological response was detected in only 59 per cent of sheep in groups III - VIII. The mean HAI titre of all animals in these groups at this time was 1/18 and there were no statistically significant differences between the mean antibody titres of each group. A comparison of the mean HAI titres of groups I - VIII at 7, 14, 21, 28 and 84 days following initial (group I) and second (groups II - VIII) injection of vaccine is shown in Table 3.2. The statistical differences between the mean titres of group I and groups II - VIII sheep and the numbers of sheep responding to vaccination are also indicated. The full results are tabulated in Appendices 3.2 and 3.3.

Following injection of a single dose of vaccine an antibody response was detected in only 4 sheep of group I and the maximum mean antibody titre was 1/17. Eight of the group II sheep responded to revaccination after an interval of one week with a maximum mean antibody titre of 1/29. There was no significant difference in the response of these sheep and the response of group I sheep.

The secondary serological responses in groups III - VIII developed quicker and to higher titres than the primary response in group I sheep or the response in group II sheep. At 7, 14, 21, 28 and 84 days following revaccination antibody titres significantly greater than those of group I were detected in groups III, V and VI. The highest mean antibody titres were consistently demonstrated in sheep of group V with a maximum mean titre of 1/724 being attained 14 days following revaccination. Following administration of a

second dose antibody titres significantly greater than those of group I were detected on all sampling days except day 84 in groups VII and VIII and only on days 7 and 21 in group IV. The control sheep of group IX remained seronegative throughout the experiment.

Several of the 80 vaccinated sheep had either remained seronegative throughout the experiment or had failed to produce a persistent antibody response. Two such animals were chosen from each of the vaccination groups I - VIII and together with the 10 control unvaccinated animals from group IX were challenged with live virus. The results are given in the next section.

Administration of different dose volumes

The HAI antibody responses 21 days after the first dose of vaccine and 28 days following the second dose of vaccine in all groups are tabulated in Table 3.3.

Twenty-eight days after revaccination a serological response was detected in 50, 83, 67 and 83 per cent of animals in group A, B, C and D respectively with corresponding mean antibody titres of 1/79, 1/50, 1/20 and 1/79.

There were no statistically significant differences between the mean antibody titres of group A, B, C or D either at 21 days after primary vaccination or at 28 days after revaccination.

CHALLENGE OF VACCINATED SHEEP WITH LIVE VIRUS

On the 16 sheep chosen from the vaccination groups I - VIII (Experiment 1) HAI antibody titres immediately prior to challenge were <10 in 11 and 1/10 or 1/20 in the remaining 5.

Clinical response

Ataxia was observed in 4 of the 10 unvaccinated control sheep (X333, X538, V875 and V876), 8 days after challenge. In two of these

affected sheep (X538, V875) symptoms progressed to death by 48 hours. No signs of clinical infection were observed in any of the vaccinated animals.

Viraemia

Virus was detected in the plasma of all but one of the 10 unvaccinated control sheep, in 9 of the 11 vaccinated but seronegative animals and in 2 of the 5 vaccinated animals that had seroconverted to maximum HAI titres of 1/10 or 1/20 (Table 3.4). The course of viraemia in the unvaccinated animals, the seronegative vaccinates and the seropositive vaccinates following challenge is illustrated in Fig. 3.1. Mean levels of viraemia were consistently highest in the unvaccinated controls and lowest in the vaccinated seropositive animals. Maximal titres of circulating virus were found on days 3 and 4 following challenge, ranging from 1.1 $\log_{10}$pfu/ml to 4.5 $\log_{10}$pfu/ml in the vaccinated sheep and 1.6 $\log_{10}$pfu/ml to 6.9 $\log_{10}$pfu/ml in the unvaccinated controls. The vaccinated animal with the maximum virus titre of 4.5 $\log_{10}$pfu/ml had been selected from vaccination group I and had received a single dose of vaccine only. Viraemia was not detected subsequent to day 5 in the vaccinated animals and day 6 in the unvaccinated controls. The highest virus concentrations were detected in the 2 unvaccinated animals that died and virus was recovered from the brains of both these animals. The full data are tabulated in Appendix 3.4.

The duration of viraemia was shorter and the magnitude of circulating virus lower in the vaccinated animals than in the control animals.

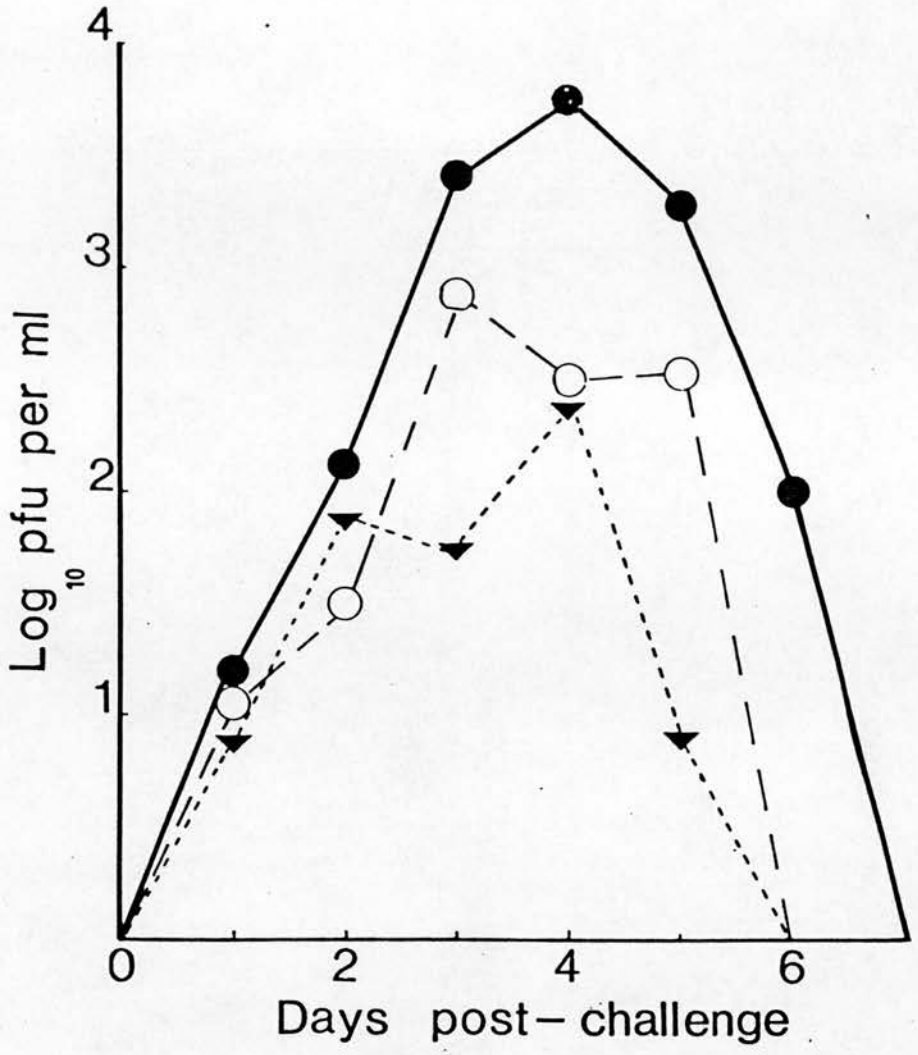
Figure 3.1

Comparison of the geometric mean titres of viraemia following peripheral inoculation with louping-ill virus in:

(i) vaccinated sheep with reciprocal antibody titres of <10 (—○—)

(ii) vaccinated sheep with reciprocal antibody titres of ≥ 10 (--▼--)

(iii) unvaccinated sheep (—●—)



●—	80	80	80	90	60	10
—○—	27	55	64	64	36	
---▼---	40	20	40	20	20	

% of animals viraemic

Antibody response

The development of HAI antibody following challenge in the unvaccinated control animals, the seronegative vaccinates and the seropositive vaccinates is illustrated in Fig. 3.2. The mean HAI titres and the number of animals responding is given in Table 3.5. The appearance of antibody correlated with a decline in circulating virus. A rise in HAI antibody was first detected in 8 of the 16 vaccinated animals on day 5 following challenge. Thereafter antibody titres increased in all animals and reached a maximum on day 9. The antibody response was initially lower in the four animals selected from vaccination groups I and II. Circulating HAI antibody developed more slowly in the unvaccinated controls and was not detected until the sixth day after challenge. Antibody titres then rose rapidly, and peak values were attained on day 12.

Initial antibody activity in all animals was due to IgM. By day 10 antibody activity in the 16 vaccinated animals was almost totally attributable to IgG whereas a substantial amount of IgM antibody activity could still be detected 14 days following challenge in the control unvaccinated animals. The full results are given in Appendix 3.5.

The development of HAI antibody was quicker and mean titres higher in the vaccinated animals than in the unvaccinated control animals.

DISCUSSION

Only 40 per cent of the animals vaccinated with a single dose of commercial vaccine produced a detectable serological response and the maximum mean antibody titre of these animals was 1/17. Revaccination

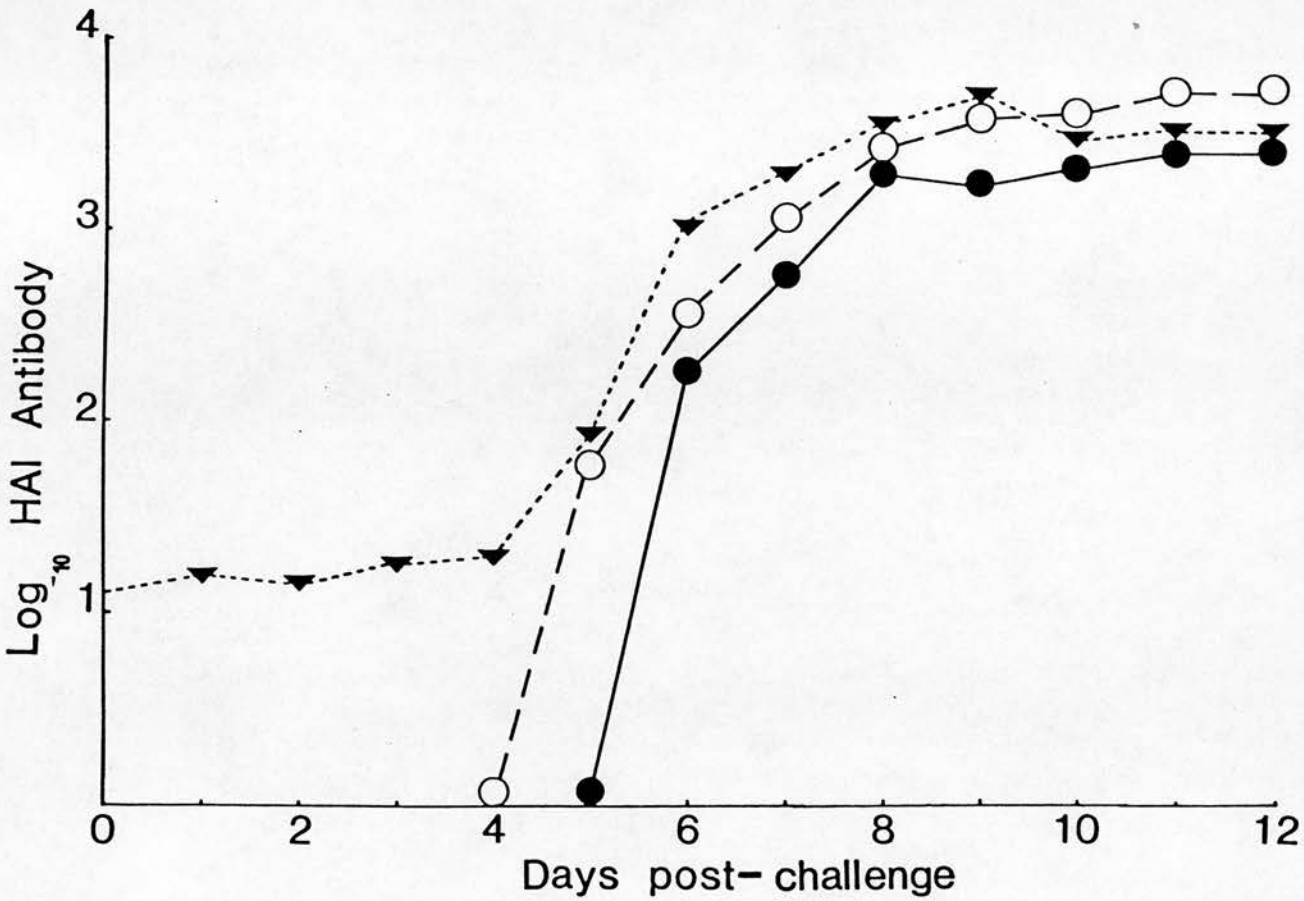
Figure 3.2

Comparison of the geometric mean reciprocal HAI antibody titres following peripheral inoculation with louping-ill virus in:

(i) vaccinated sheep with reciprocal antibody titres of <10 (—O—)

(ii) vaccinated sheep with reciprocal antibody titres of ≥ 10 (--▼--)

(iii) unvaccinated sheep (—●—)


4
3
2
1
Log2 HAI Antibody
0
2
4
6
8
10
12
Days post-challenge

one week after the initial dose failed to increase antibody titres significantly. Revaccination at 2, 3, 4 and 8 weeks produced typical secondary immune responses with antibody titres significantly higher than those detected in sheep which received one dose of vaccine and those revaccinated after one week. Administration of a second dose of vaccine at 16 or 24 weeks significantly accelerated the rise in antibody titres but these declined rapidly. The highest mean antibody titres were consistently demonstrated in sheep revaccinated after an interval of 4 weeks.

Two 1ml doses of commercial vaccine with an interval of 2 - 8 weeks between injections were therefore shown to be necessary to provide a satisfactory level of immunity against infection with live virus.

Although all 16 vaccinated animals were protected from clinical disease following challenge with live virus, circulating virus was detected in 11. Of these 11, 2 had pre-challenge HAI titres of 1/10 and the remainder were seronegative. Viraemia was not detected in the remaining 5 animals. Of these animals 2 had pre-challenge HAI titres of 1/20, one had a pre-challenge titre of 1/10, and the remaining 2 were seronegative. These animals did however become infected as judged by the rise in antibody titre following challenge. Protection against challenge is therefore afforded to animals with a vaccinal HAI titre of 1/10 and circulatory virus is inhibited to undetectable levels in animals with an HAI titre of 1/20.

Results obtained from protection studies in lambs that had acquired passive immunity to louping-ill (Reid and Boyce, 1976) were similar to those reported here. Lambs with low detectable titres of

maternal antibody experienced infection following challenge but in the absence of a detectable viraemia, whilst lambs in which maternal antibody had declined to undetectable levels developed viraemias of low intensity.

In studies on the susceptibility of sheep to sub-cutaneous inoculation with live louping-ill virus, Reid and Doherty (1971b) suggested a relationship between the magnitude and duration of viraemia, the rate of the serum antibody response and survival. They demonstrated that the intensity of viraemia was lower and of shorter duration and serum antibody developed quicker in surviving animals compared to animals succumbing to infection. Similarly in this study, following challenge with live virus the magnitude and duration of viraemia is reduced and the development of humoral antibody quicker in vaccinated animals compared to both the surviving unvaccinated control animals and those succumbing to infection. It is suggested therefore that although vaccination may fail to consistently produce a detectable serological response it does however sensitize the immune system to viral antigen and thus produce an accelerated antibody response in the event of subsequent exposure to virus.

Table 3.1 The geometric mean reciprocal HAI titres and the number of animals responding to primary vaccination.

Group	Interval (weeks) between vaccine doses	Response 7 days following primary vaccination	Response immediately prior revaccination
I	Nil	8.7 ± 1.60* 2/10	-
II	1	5 0/10	5 0/10
III	2	5 0/10	26 ± 1.5 7/10
IV	3	5.8 ± 1.14 1/10	11 ± 1.4 4/10
V	4	5.8 ± 1.14 1/10	32 ± 1.5 9/10
VI	8	5.8 ± 1.14 1/10	28 ± 1.9 5/10
VII	16	5 0/10	11 ± 1.6 4/10
VIII	24	5 0/10	10 ± 1.2 7/9

*Mean $\pm$ SE

Table 3.2 The haemagglutination-inhibiting antibody response in sheep following vaccination with louping-ill vaccine

Group	Intervals between vaccine doses (weeks)	Days following vaccination				
		7	14	21	28	84
I	nil	[x] 8.7 [xx] 2/10	17 4/10	14 4/10	11 3/10	17 4/10
II	1	20 4/9	20 8/9	22 5/9	15 4/9	29 8/9
III	2	138*** 9/10	129* 9/10	91* 8/10	120** 9/10	120* 9/10
IV	3	98** 8/10	85 8/10	120* 8/10	49 7/10	32 6/10
V	4	447*** 10/10	724*** 10/10	223** 9/10	417*** 10/10	138* 9/10
VI	8	138*** 9/10	138* 9/10	112* 9/10	105** 7/10	158* 8/10
VII	16	98** 9/10	105* 10/10	91* 10/10	363*** 10/10	21 4/10
VIII	24	158*** 9/9	137* 9/9	468*** 9/9	170*** 9/9	18 7/9

[x] The geometric mean reciprocal HAI titre following vaccination (Group I) and revaccination (Groups II - VIII)

[xx] The number of animals responding to vaccination/number of animals vaccinated.

The statistical difference of the titres between Group I and any group is indicated as follows:-

* $p < 0.050$

** $p < 0.010$

*** $p < 0.001$

Table 3.3 The geometric mean reciprocal HAI titres and the number of animals responding to a single and double dose of varying volumes of vaccine

Group	Vaccine* Dose	Response 21 days post primary vaccination	Response 28 days post revaccination
A	1ml + 1ml	11 ± 1.7 2/6	79 ± 2.5 3/6
B	1ml + 0.5ml	16 ± 1.5 4/6	50 ± 1.8 5/6
C	0.5ml + 0.5ml	5 0/6	20 ± 1.9 4/6
D	0.25ml + 0.25ml	7 ± 1.4 1/6	79 ± 2.7 5/6

*Second dose of vaccine administered 21 days following primary vaccination

Mean $\pm$ SE

Table 3.4 Mean titres* of virus (log_{10}pfu/ml) and the number of animals circulating virus following challenge

Sheep	Days post challenge					
	1	2	3	4	5	6
Unvaccinated controls	1.2 ± 0.18 8/10	2.1 ± 0.32 8/10	3.4 ± 0.44 8/10	3.7 ± 0.62 9/10	3.3 ± 0.52 6/10	2.0 1/10
Vaccinates: titre <10 at challenge	1.2 ± 0.12 3/11	1.5 ± 0.17 6/11	2.9 ± 0.38 7/11	2.5 ± 0.48 7/11	2.6 ± 0.70 4/11	
Vaccinates: titre >10 at challenge	0.9 ± 0.50 2/5	1.9 1/5	1.8 ± 0.85 2/5	2.4 1/5	0.9 1/5	

Mean titres ± SE of virus levels in those sheep circulating virus

Table 3.5 Mean reciprocal HAI titres* (log_{10}) and the number of animals with a detectable serological response following challenge with live virus

Sheep	Days post challenge							
	5	6	7	8	9	10	11	12
Unvaccinated controls		± 2.2 0.16 9/10	± 2.7 0.18 10/10	± 3.3 0.12 10/10	± 3.3 0.15 10/10	± 3.4 0.10 10/10	± 3.4 0.15 10/10	± 3.5 0.16 10/10
Vaccinates: titre <10 at challenge	± 1.8 0.20 5/11	± 2.5 0.15 11/11	± 3.0 0.12 11/11	± 3.3 0.11 11/11	± 3.6 0.14 11/11	± 3.6 0.09 11/11	± 3.7 0.11 11/11	± 3.7 0.09 11/11
Vaccinates: titre ⩾10 at challenge	1.9 3/5	± 3.0 0.15 5/5	± 3.3 0.07 5/5	± 3.5 0.17 5/5	± 3.6 0.11 5/5	± 3.6 0.20 5/5	± 3.5 0.18 5/5	± 3.5 0.16 5/5

*Mean titres ± SE of sera of those animals producing a detectable serological response

CHAPTER FOUR

GROWTH OF LOUPING-ILL VIRUS IN BHK-21 CELLS

INTRODUCTION

Propagation of louping-ill virus was first demonstrated by Rivers and Ward (1933) using minced chick embryo tissue suspended in Tyrodes solution. Wilson (1945) also using this method showed that virus titres were maximum 3-4 days following infection. The replication of louping-ill virus in embryonated hens' eggs has been reported by Burnet (1936) and Edward (1947a). Edward (1947a) demonstrated that embryos at 6-10 days incubation were most susceptible to virus and that following inoculation either into the yolk sac or the embryo, the latter contained the higher concentration of virus. Titres of virus were highest 3-4 days after inoculation. The propagation of louping-ill virus in monolayer tissue culture by other workers is summarised in Table 4.1.

Early attempts to produce vaccines from tissue culture propagated virus were unrewarding (Wilson, 1945). However Brotherston and Boyce (1970) developed an experimental louping-ill virus vaccine based on propagation of the virus in secondary sheep kidney cells. For commercial production of this vaccine virus is cultured in the continuous BHK-21 clone 13 cell line (Appendix 3.1).

This chapter records an examination of the growth of louping-ill virus in BHK-21 cells in an attempt to define optimal conditions for virus replication.

Part I examines factors affecting the adsorption of virus. Polyions such as Diethylaminoethyl-Dextran (DEAE-D) have been used

extensively in attempts to facilitate adsorption of virus to cells and thus increase virus infectivity (Bijlenga and Van Den Bogaard, 1973; Shigeko, 1968; Rossi, 1971; McCutchan and Pagano, 1968; Stow and Wilkie, 1976) and pretreatment of cell monolayers with dimethyl-sulphoxide (DMSO) has been reported to enhance plaque formation (Cleaver, 1974). The effect of pretreating BHK-21 cells with DEAE-D and DMSO on virus adsorption was therefore examined.

Poste (1970, 1972) and Poste and Allison (1971) proposed that an essential step in adsorption is the displacement of calcium ions from sites within the surface membrane. Volkert and Horsfall (1947) studying pneumonia virus of mice showed that the presence of trypsin inhibited virus cell attachment. The effect of adsorption of louping-ill virus to cell suspensions prepared without the use of trypsin and a chelating agent was thus also investigated.

Part II examines the factors affecting growth of virus. Contamination of cell cultures with mycoplasmas can cause either an increase in virus yields (Singer, Kirschstein and Barile, 1969) a decrease in virus yields (Butler and Leach, 1964; Singer, Fitzgerald, Barile and Kirschstein, 1970) or no appreciable effect (Reed, 1971). The growth of louping-ill virus both in mycoplasma-free and mycoplasma-contaminated BHK-21 cells was therefore examined. The growth of louping-ill virus in various types of culture vessels incubated both as stationary and rolling cultures in BHK virus growth media and serum-free media was also examined.

Viral infectivity of harvests grown in various types of culture bottles was compared and their application for large scale virus production discussed.

PART I ADSORPTION OF LOUPING-ILL VIRUS TO BHK-21 CELLS

MATERIALS AND METHODS

Cells were obtained by trypsinization of 3-day-old BHK-21 cell cultures and counted using a Mod-Fuchs Rosenthal counting chamber. Virus inoculum Li/31/M5 BHK/2 was prepared as described in Chapter Two. Adsorption was carried out by either mixing virus and cells together in suspension or by inoculating confluent cell monolayers.

ADSORPTION OF VIRUS TO CELLS IN A CELL SUSPENSION

Cell suspensions were prepared by resuspending trypsinized cells in BHK virus growth media. The virus cell suspensions were incubated in a water bath at 37.0 $\pm$ 0.5^{o}C and shaken at 15-minute intervals throughout the adsorption period. Virus was incubated in an identical fashion but without the addition of cells. Aliquots were removed from the virus cell suspension throughout adsorption, centrifuged at 750g for 15 minutes at 4.0 $\pm$ 0.5^{o}C and the deposited cells washed twice in PBS and finally resuspended in 1ml of PK/BA media. The infected cell suspensions and cell-free supernates were diluted and immediately assayed for infected cells or free virus using the plaque method or by direct immunofluorescence. The plaque method of assay was used on the rationale that each infected cell will, in a similar fashion as a free virus particle, become a centre of virus replication. When plated onto a cell monolayer one infected cell will produce one plaque. The number of plaques produced by a washed suspension of infected cells will thus reflect the actual number of infected cells if assay immediately follows sampling and before the cells have started to release new virus. Assay of virus infected cells by the plaque method and immunofluorescence were compared.

ADSORPTION OF VIRUS TO CELLS IN A CELL MONOLAYER

Confluent cell monolayers were prepared by seeding Pyrex glass tubes (15 x 150mm) with 10^6 cells in 1.5ml of BHK cell growth media and incubating for 19 hours at 37.0 $\pm$ 0.5^{o}C on a roller drum rotating at 0.2 rpm. The cells from 10 tubes were then trypsinized and counted and the media poured from the remaining tubes. The cell monolayers were inoculated with 1ml of virus diluted in BHK virus growth media to give the required input multiplicity. The tubes were incubated on a roller drum at 37^{o}C for up to 90 minutes. At intervals throughout adsorption 10 or more tubes were randomly selected, the virus inoculum poured off and the cells removed by trypsinization. The trypsinized cells were pooled, sedimented by centrifugation at 750g, washed twice in PBS and finally resuspended in 1ml of PK/BA media. The cell suspensions were assayed for infected cells using the plaque method.

Assay of infected cells by the plaque method

The plaque assay method used was as described in Chapter Two with the exception that the 2 hour adsorption period prior to addition of overlay was reduced to 30 minutes.

Assay of infected cells by direct immunofluorescence

The direct immunofluorescence technique used is described in Chapter Two.

RESULTS

1. COMPARISON OF THE PLAQUE METHOD AND DIRECT IMMUNOFLUORESCENCE FOR THE ASSAY OF INFECTED CELLS

BHK-21 cells suspended in BHK virus growth media were mixed with virus inoculum to give an added multiplicity of 9 pfu/cell and 13 pfu/cell with a final cell concentration in the adsorbing suspension

of 10^7 cells/ml. An aliquot was removed from each cell-virus suspension after 90 and 120 minutes respectively of adsorption and assayed in duplicate for infected cells using the plaque method. The remaining cell-virus mixtures were washed twice to remove free unadsorbed virus and the cells resuspended in BHK virus growth media. Pyrex glass tubes containing coverslips were inoculated with 1.5×10^6 adsorbed cells and incubated as stationary cultures at 37^oC. Coverslips removed between 10 and 11 hours post infection and treated with FITC conjugated sheep anti- louping-ill globulin showed specific fluorescence in a proportion of cells. The fluorescence was weak and localised around the cell nucleus. The percentage of these infected cells was determined as described in Chapter Two. Coverslips removed between 11 and 24 hours post infection showed strong fluorescence throughout the cytoplasm of all cells (Fig. 4.1).

With an adsorption period of 2 hours and an added multiplicity of 9, 9 per cent of cells were infected and with a multiplicity of 13, 14.6 per cent of cells were shown to be infected using the plaque assay method. The plaques were indistinguishable from plaques produced by free virus particles. With an input multiplicity of 9 and 13 the percentage of infected cells determined by immunofluorescence was 7.3 and 8.4 respectively. The results were compared statistically using the Mann-Whitney non-parametric test. The plaque assay method detected a significantly higher ($p < 0.05$) number of infected cells than the immunofluorescence technique. In the following studies the plaque method was routinely used for assay of infected cells.

Figure 4.1

Immunofluorescent staining of BHK-21 cells infected with louping-ill virus

(a) 10-12 hours post infection

(b) 21-24 hours post infection

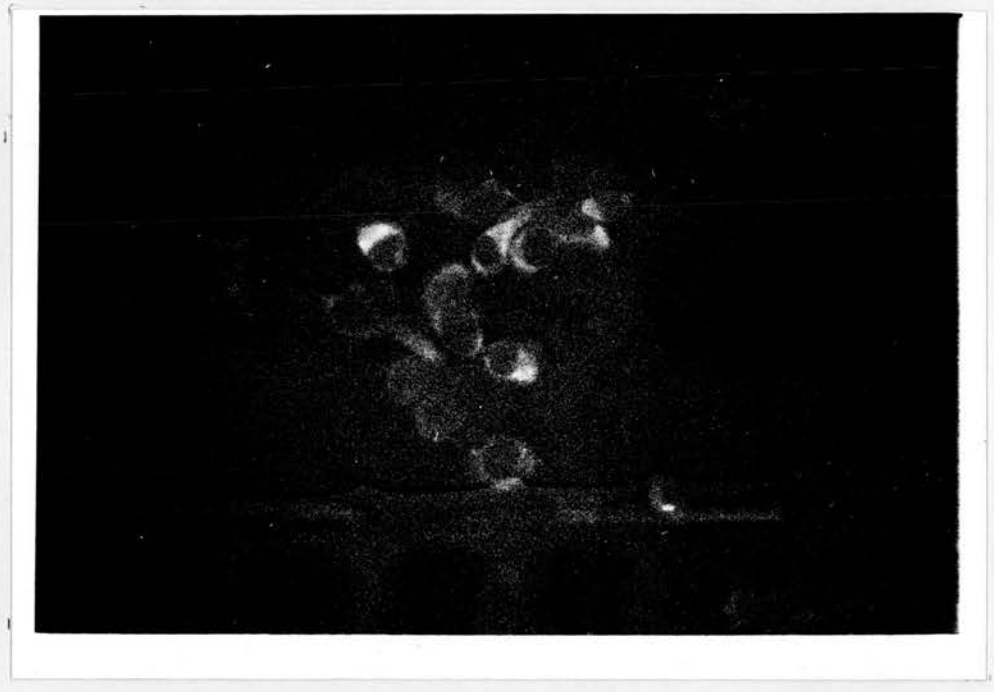


Fig. 4.1a

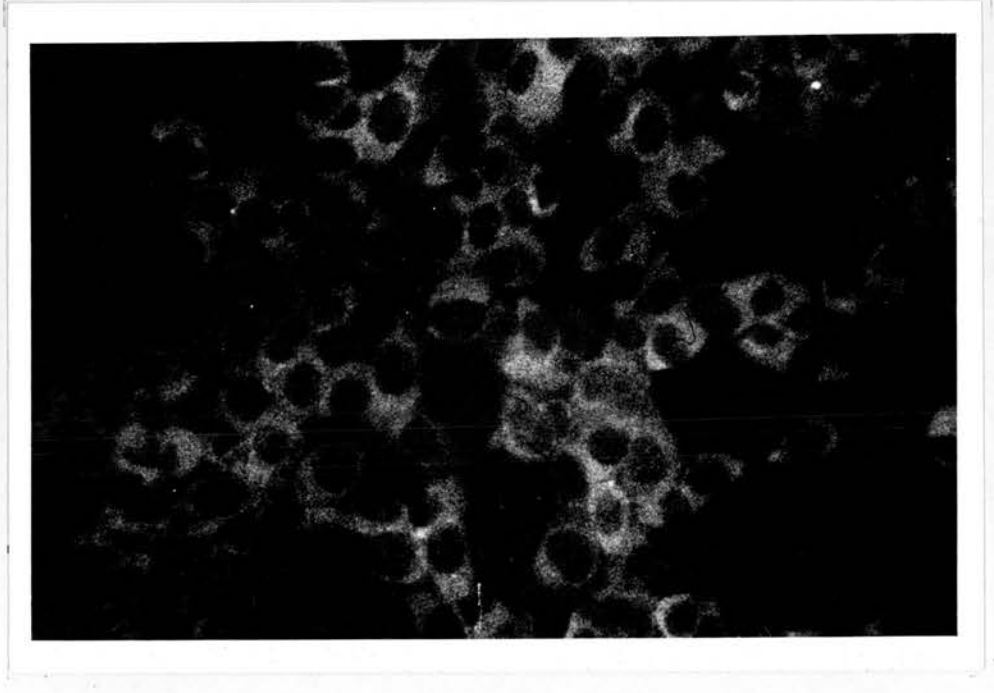


Fig. 4.1b

2. ADSORPTION OF VIRUS TO CELLS IN A CELL SUSPENSION

(i) The effect of the multiplicity of infection and the concentration of cells on virus adsorption

BHK-21 cells suspended in BHK virus growth media were mixed with virus to give (a) an added multiplicity ranging from 0.09 to 95 pfu/cell and, (b) a cell concentration of between 10^6 and 10^8 cells/ml with a constant multiplicity of 1.8 pfu/cell.

With an added multiplicity of one and a cell concentration of 5×10^6 cells/ml the percentage of infected cells after 60 minutes adsorption at 37°C was 0.35. Unadsorbed virus was detected in the cell-free supernatants. Incubation of the virus cell mixture for a further 60 minutes did not appreciably increase adsorption. Thermal degradation of virus was not detectable throughout the adsorption period. At input multiplicities of 0.09, 5 and 95 pfu/cell and cell concentrations of 10^7/ml, 5×10^6/ml and 10^6/ml, 0.38, 2.0 and 4.0 per cent respectively of cells were infected after adsorption for 120 minutes. With a constant input multiplicity of 1.8 pfu/cell and a cell concentration of 7.5×10^7/ml, 3.8×10^7/ml, 10^7/ml and 3.8×10^6/ml, 13, 13, 8 and 5 per cent respectively of cells were infected after 60 minutes adsorption. There was a linear relationship between the percentage of infected cells and the cell concentration, the correlation coefficient being 0.99 ($p < 0.01$) (Fig. 4.2). The final concentration of serum in each adsorbing suspension was constant at 10 per cent.

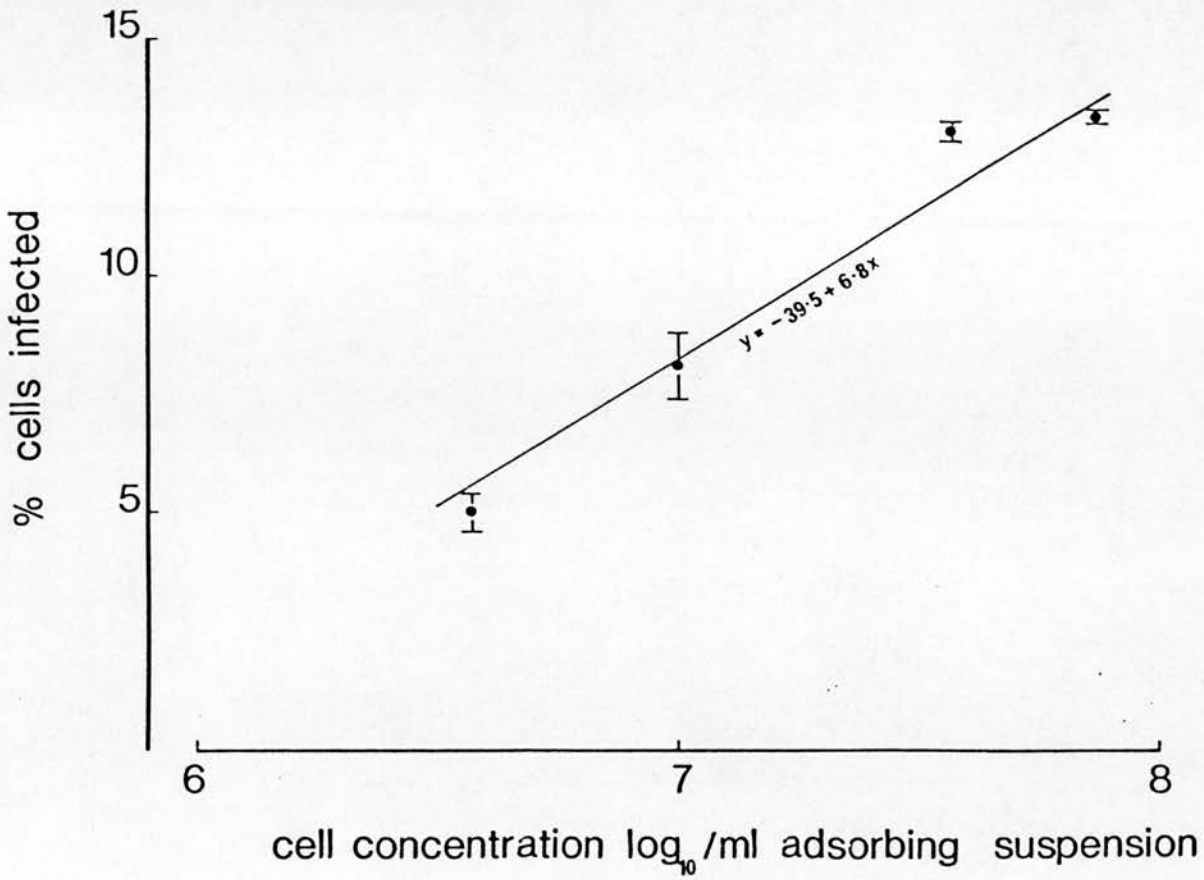
(ii) Preparation and pretreatment of cell suspensions

Preparation of cell suspensions

Cell suspensions were routinely prepared by trypsinization of

Figure 4.2

The effect of cell concentration on the probability of virus adsorption


15
10
5
% cells infected
y = −39·5 + 6·8x
6
7
8
cell concentration log10/ml adsorbing suspension

cell monolayers using a mixture of trypsin and versene. To investigate the possibility of deleterious effects brought about by the use of trypsin and a chelating agent single cell suspensions were prepared either by mechanical detachment using a rubber policeman or by trypsinization. Adsorption of virus to each cell suspension was compared. With an added multiplicity of 3.5 pfu/cell and a cell concentration of 5×10^5 cells/ml, 0.2 per cent of the trypsinized cells were infected compared with 0.38 per cent of cells prepared by mechanical detachment.

Pretreatment of cell suspensions

To determine the effect of pretreating cells with DEAE-D and DMSO on viral adsorption, 9 aliquots of cells were prepared and resuspended in BHK cell growth media or BHK cell growth media containing DEAE-D at concentrations of 5 µg/ml, 15 µg/ml, 25 µg/ml and 50 µg/ml or DMSO at concentrations of 0.5, 2, 5 and 10 per cent (v/v). Suspensions were incubated at $37^{\circ}C$ for 60 minutes, the cells then sedimented by centrifugation at 750g and washed twice with media. All cell suspensions were adjusted to 5×10^6 cells/ml and the percentage of infected cells assayed after incubation for 60 minutes. The input multiplicity was 13 pfu/cell. The results are shown in Table 4.2. The percentage of untreated cells that were infected was 1.7 compared to 2.7 per cent of cells pretreated with 25 µg/ml DEAE-D and 3.2 per cent of cells pretreated with 10 per cent DMSO.

(iii) The effect of serum on virus adsorption to BHK-21 cells and pig kidney cells.

Effect of serum on virus adsorption to BHK-21 cells

Eight aliquots of trypsinized BHK-21 cells were resuspended in

6 different media varying in serum content and concentration. Sheep serum and adult bovine serum were inactivated prior to use by heating at 56^{o}C for 35 minutes. Two ml of virus inoculum were mixed with 8ml of each cell suspension to give an input multiplicity of 10 pfu/cell and 1 pfu/cell and a cell concentration of 10^{6} cells/ml and 6×10^{6} cells/ml respectively. After 60 minutes of adsorption an aliquot was removed from each cell-virus suspension and assayed (in duplicate) for the number of infected cells using the plaque assay method.

The percentage of cells infected and the statistical differences in the number of cells infected with 8 per cent LS in the adsorbing suspension compared to the number infected in each of the other adsorbing suspensions are shown in Table 4.3. There was no significant difference in virus adsorption with 8 per cent LS or 8 per cent FBS in the adsorbing suspension (input multiplicity of 10 pfu/cell). However the numbers of infected cells were significantly higher with 4 per cent LS ($p < 0.001$), 8 per cent NBCS ($p < 0.001$), 8 per cent ABS ($p < 0.01$), and 8 per cent SS ($p < 0.05$) in the adsorbing suspension. Adsorption was most efficient in the presence of 4 per cent LS and 8 per cent NBCS (24 and 23 per cent of cells respectively were infected) The data were analysed using an analysis of variance. With a multiplicity of one, virus adsorption was greater in media containing 8 per cent ABS (8 per cent of cells infected), than in media containing 8 per cent LS (one per cent of cells infected).

Adsorption of virus to pig kidney cells

The IB/RS2 pig kidney (PK) cell line was used to compare virus adsorption to BHK-21 cells and PK cells. The effect of serum and

cell pretreatment on the efficiency of virus adsorption to PK cells was examined. PK cell suspensions were prepared by trypsinization of 4-day-old confluent cell monolayers grown in PK cell growth media containing 10 per cent ABS. Cells were pretreated with 10 per cent DMSO as described previously. BHK-21 cells suspended in BHK virus growth media and PK cells suspended in PK virus growth media containing 10 per cent ABS were each adsorbed with virus. With an input multiplicity of 2.8 pfu/cell and a cell concentration of 6×10^6 cells/ml 2.8 per cent of untreated and 2.8 per cent of treated BHK-21 cells were infected compared with 16 per cent of untreated and 20 per cent of treated PK cells.

To determine the effect of serum on virus adsorption to PK cells, confluent PK cell monolayers were trypsinized and the cells resuspended in fresh growth media containing 10 per cent ABS or 10 per cent LS. After a further 24 hours incubation the resultant monolayers were retrypsinized and the cells resuspended in media containing the appropriate sera. Virus and each cell suspension were mixed to give an added multiplicity of 13.6 : 1 and a cell concentration of 6×10^6 cells/ml. After 60 minutes of adsorption an aliquot was removed from each cell-virus suspension and assayed (in triplicate) for the number of infected cells using the plaque assay method. The data were statistically analysed using a two-sample t-test. The mean number of cells infected (1.6×10^6 pfu/ml) in a suspension containing a final concentration of 8 per cent ABS was significantly higher ($p < 0.01$) than the number infected (4.8×10^5 pfu/ml) in a suspension containing a final concentration of 8 per cent LS.

Fractionation of whole lamb serum: adsorption in the presence of three serum fractions

The serum in the growth media specified for virus adsorption and virus growth in the preparation of louping-ill virus vaccine is necessarily of ovine origin. To investigate further the apparent inhibitory effect of lamb serum on virus adsorption, a 3ml volume of whole lamb serum was applied to a Sephadex G200 2.5cm x 8.5cm column equilibrated with 0.1 M Tris HCl and 1M sodium chloride pH 8 buffer. A flow rate of 17ml/hour was applied and the eluate fractions collected at one minute intervals. Protein present in the eluate fractions was continuously monitored using a LKB Uvicord II* reading absorbancy at 280nm, connected to a Vitatron linear recorder (Type UR404**). Three distinct protein peaks emerged (Fig. 4.3). The eluate fractions corresponding to each peak were pooled and the resulting 3 fractions dialysed against polyethylene glycol 6000 for 18 hours and then against BHK cell growth media without serum overnight. The volume of each was made up to 6ml with cell growth media minus serum. BHK-21 cell suspensions were prepared by trypsinizing 3-day-old confluent monolayers and resuspending the cells in BHK virus growth media containing (i) 10 per cent LS, (ii) 5 per cent LS, and (iii) fractions 1, 2 and 3 at a concentration of 20 per cent. Virus (2ml) and cell suspensions (8ml) were mixed to give a multiplicity of 3 pfu/cell and a cell concentration of 10^7 cells/ml. After 60 minutes of adsorption an aliquot was removed

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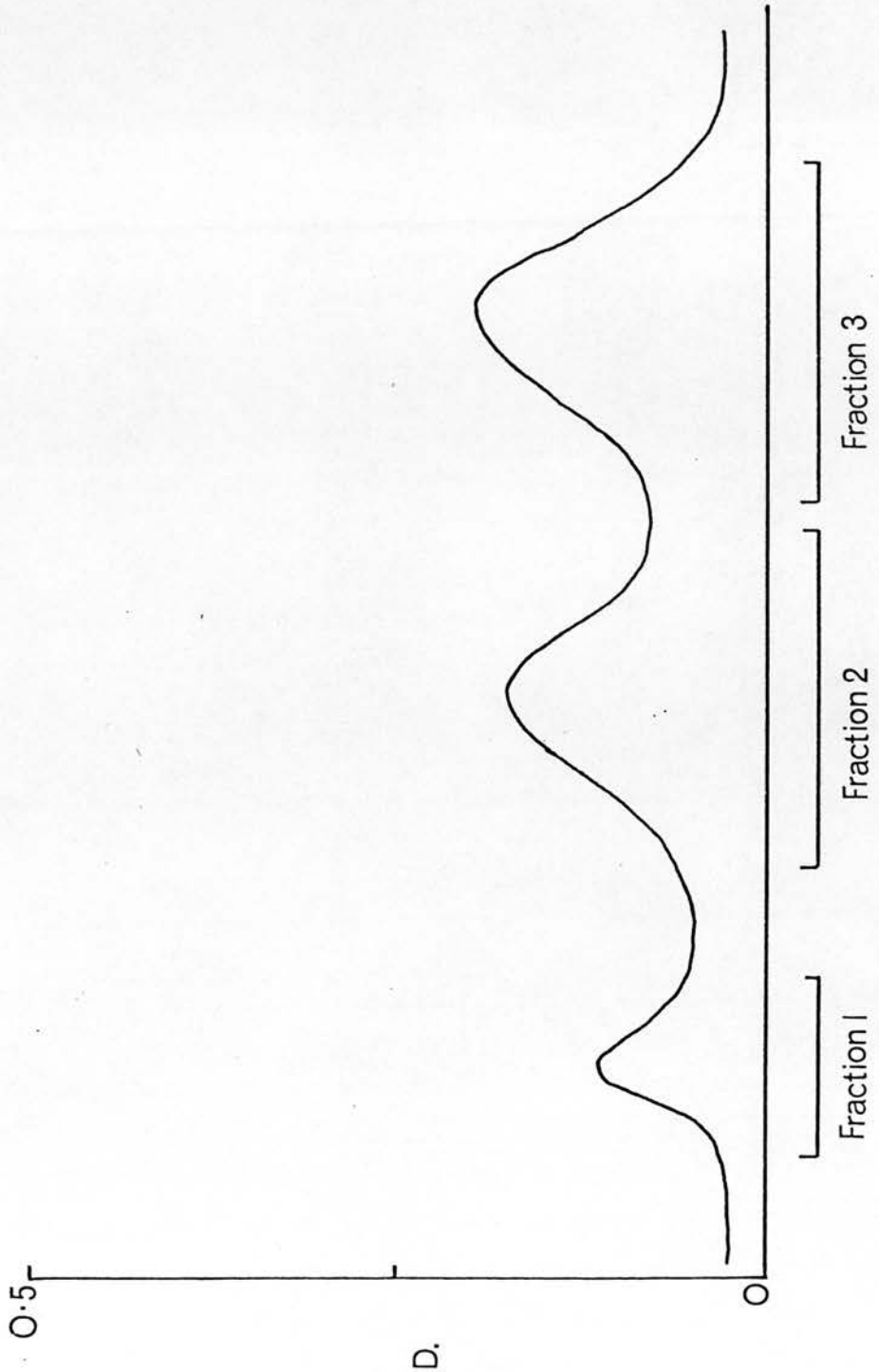
* LKB Instruments Ltd., Bromma, Sweden.

**Vitatron Dreren, Holland.

Figure 4.3

Protein distribution of normal lamb serum following fractionation through a Sephadex G200 column


0·5
O
O.D.
Fraction I
Fraction 2
Fraction 3

from each cell-virus suspension and assayed (in duplicate) for the number of infected cells using the plaque assay method.

The statistical differences between the number of cells infected in a cell-virus suspension with a final concentration of 8 per cent complete serum and the number infected with a serum content of 4 per cent complete and 15 per cent fractionated serum (final concentrations) are shown in Table 4.4. The mean number of cells infected were significantly higher with a 4 per cent complete (7.2×10^5 pfu/ml, $p < 0.001$), fraction 3 (1.9×10^6 pfu/ml, $p < 0.001$), fraction 2 (6.8×10^5 pfu/ml, $p < 0.01$) and fraction 1 (3.4×10^5 pfu/ml, $p < 0.05$) serum content in the adsorbing suspension compared to the mean number infected with a serum content of 8 per cent complete (2.0×10^5 pfu/ml). The data were analysed using an analysis of variance.

An aliquot of complete serum was screened for haemagglutination-inhibiting antibody activity to louping-ill virus using the method described in Chapter Two. No activity either in the IgM or IgG antibody class was detected.

Resuspension of BHK-21 cells in the virus inoculum

The efficiency of adsorption of virus to cells by directly resuspending the cells in virus was determined. Confluent BHK-21 cell monolayers were trypsinized, the cells washed and resuspended in the virus inoculum to give input multiplicities of 2.5 pfu/cell, 4.4 pfu/cell and 8.6 pfu/cell with cell concentrations of 4×10^7 cells/ml, 3×10^7 cells/ml and 2×10^7 cells/ml respectively. The best adsorption (46-56 per cent of cells infected) occurred when the cells were resuspended directly in the virus inoculum with an input multiplicity of 8.6 pfu/cell (Table 4.5).

3. ADSORPTION OF VIRUS TO BHK-21 CELLS IN CELL MONOLAYERS

Confluent cell monolayers were inoculated with one ml of a virus suspension to give an input multiplicity of 15 pfu/cell. After 90 minutes adsorption 0.6 per cent of cells were shown to be infected.

The effect of pretreating cells in a monolayer with 10 per cent DMSO was also investigated. The procedure was as described earlier except that prior to inoculation of the monolayer the media was decanted from half of the tubes and replaced with BHK cell growth media plus 10 per cent DMSO. After a further 60 minutes incubation the media was removed from all tubes and one ml of a virus suspension added to each tube to give a multiplicity of 1.5 pfu/cell. After 60 minutes adsorption 0.1 per cent of untreated and 0.24 per cent of pretreated cells were infected.

DISCUSSION

The plaque method was shown to be a significantly more sensitive method for assaying the proportion of cells infected following adsorption than the immunofluorescence method. Plaques were indistinguishable from those produced by free virus and non-infected cells did not appear to interfere with the assay.

Adsorption of virus to BHK-21 cells and the IB/RS2 PK cell line was shown to be inhibited by the presence of lamb serum in the adsorbing mixture and on further investigation it was apparent that adsorption was specifically inhibited by a component or components of high molecular weight present in the first and, to a lesser extent, second protein peaks following Sephadex-G200 fractionation of whole serum. The serum was screened for HAI antibody activity to louping-ill

virus and no activity either in the IgM or IgG antibody class could be detected. It is unlikely therefore that the adsorption inhibiting factor was a specific immunoglobulin against louping-ill. A visna virus inhibiting factor (VIF) present in normal sheep sera and cerebrospinal fluid has been reported by Thormar, Wisnieski and Lin (1979). They showed that the VIF was not an immunoglobulin with specific antibody against visna virus and suggested that it may be a lipoprotein. The growth of visna virus was shown to be totally inhibited in sheep cell monolayers containing 90 per cent normal sheep serum. Under the experimental conditions described here the louping-ill virus inhibiting factor present in normal lamb serum appears to be specific to virus attachment and adsorption as replication of virus in cells grown in media containing 10 per cent lamb serum was unaffected (Part II). Different batches of lamb serum were used throughout the study and it is possible that a variation in the degree of adsorption inhibition existed between batches.

Adsorption of louping-ill virus to BHK-21 cells was shown to be dependent on the cell concentration and virus input. Raising the cell concentration and the added multiplicity increased the probability of adsorption and was most readily effected by resuspending cells directly in the virus inoculum. In addition under these conditions adsorption was not adversely affected by the introduction of fresh lamb serum into the adsorbing preparation. Similarly Davey, Dennett and Dalgarno (1973) studying the growth kinetics of 2 togaviruses, Semliki Forest virus and Kunjin virus, showed that increasing the cell concentration during adsorption increased the percentage of cells infected, and Rubin, Franklin and Baluda (1957) demonstrated that adsorption of

Newcastle disease virus to chick-embryo-lung epithelium approached a maximum when the total number of cells in the layer was 10^7.

It has been suggested that DEAE-D may bind to the cell surface or complex with virus particles and thus increase the efficiency of virus adsorption and virus infectivity (Rossi and Kiesel, 1978; Rossi, 1971 and Shigeko, 1968). Pretreatment of cells with DEAE-D has been shown to enhance the infectivity of respiratory syncytial virus in African green monkey kidney cells and bovine embryonic lung cultures (Shigeko, 1968; Rossi and Kiesel, 1978), the growth of rubella virus in BHK-21 cells (Vaheri, Sedwick and Plotkin, 1967) and plaque production in alphaviruses in chick embryo culture (Pattyn and Vleesschauwer, 1969). McCutchan and Pagano (1968) however reported a 20-fold reduction in the infectivity of Simian virus 40 in the presence of DEAE-D and Stow and Wilkie (1976) showed that the infectivity of Herpes Simplex virus in BHK-21 cells was unaffected by treatment with DEAE-D. Cleaver (1974) observed an increase in plaque size and numbers following addition of DMSO to the agar overlay of Simian virus 40 plaque assays. It is evident therefore that depending on the virus-cell system employed, DMSO and DEAE-D may exert either an enhancing or a depressing effect on virus adsorption. In the present study treatment of cells with these two chemicals had little effect on virus adsorption.

In conclusion, the rate of adsorption of louping-ill virus to BHK-21 cells proved to be a function of the cell concentration, the input multiplicity and the serum present in the adsorbing preparation. Adsorption was maximal after 60 minutes incubation at $37^{\circ}C$ and was

more efficient to cells in a cell suspension than to cells in a monolayer.

The influence of the efficiency of adsorption on virus growth and virus yield will be discussed in Part II.

Table 4.1 Replication of louping-ill virus in tissue culture

Cell culture	Cytopathic effect	Reference
Detroit 6	+	Oker-Blom 1956
HeLa	+	Von Zeipel + Svedmyr 1958 Levkovich + Karpovich 1962
Pig kidney	+	Williams 1958
Cynomolgus monkey heart	+	Levkovich + Karpovich 1962
Human angiosarcoma	+	Levkovich + Karpovich 1962
Hep-2	-	Levkovich + Karpovich 1962
Chick embryo	-	Levkovich + Karpovich 1962
BHK-21	+	Karabatsos + Buckley 1967
Sheep kidney	+	Brotherston + Boyce 1970

Table 4.2 Adsorption of virus to BHK-21 cells in suspension: effect of cell pretreatment

Pretreatment	pfu/ml adsorbing suspension	% cells infected
Untreated	$0.8 \pm 0.08 \times 10^5$	1.7
5 µg/ml DEAE-D	$1.2 \pm 0.07 \times 10^5$	2.5
15 µg/ml DEAE-D	$1.0 \pm 0.04 \times 10^5$	2.1
25 µg/ml DEAE-D	$1.3 \pm 0.4 \times 10^5$	2.7
50 µg/ml DEAE-D	$1.2 \pm 0.28 \times 10^5$	2.5
0.5% DMSO	$0.8 \pm 0.9 \times 10^5$	1.8
2% DMSO	$1 \pm 0.01 \times 10^5$	2.1
5% DMSO	$0.7 \pm 0.15 \times 10^5$	1.4
10% DMSO	$1.5 \pm 0.18 \times 10^5$	3.2

Pretreatment at 37°C for 60 minutes

Adsorption at 37°C for 60 minutes

Input multiplicity 13 pfu/cell, cell concentration 5×10^6 cells/ml

Table 4.3 Adsorption of virus to BHK-21 cells in suspension: the effect of serum on the probability of adsorption

Serum Content: Cell growth media	Serum Content: Virus adsorption media	Input multiplicity pfu/cell	pfu/ml‡ adsorbed cells	% cells infected
Lamb 5%	8%	10	$3.4 \pm 1.3 \times 10^5$	5.6
			$4.5 \pm 0.76 \times 10^5$	7.4
Lamb 5%	4%	10	$1.3 \pm 0.3 \times 10^{6***}$	21.5
			$1.4 \pm 0.15 \times 10^6$	23.8
Newborn calf 5%	8%	10	$1.6 \pm 0.3 \times 10^{6***}$	26.7
			$1.3 \pm 0.18 \times 10^6$	20.8
Adult bovine 5%	8%	10	$9 \pm 0.37 \times 10^{5**}$	15.0
			$9.5 \pm 0.3 \times 10^5$	15.8
Sheep 5%	8%	10	$5.4 \pm 1.1 \times 10^{5*}$	9.0
			$8.1 \pm 2.5 \times 10^5$	13.5
Foetal bovine 5%	8%	10	$2.9 \pm 0.9 \times 10^5$	4.9
			$2.8 \pm 0.5 \times 10^5$	4.7
Lamb 5%	8%	1	$0.6 \pm 0.2 \times 10^5$	1.0
Adult bovine 5%	8%	1	$4.7 \pm 1.3 \times 10^5$	7.8

* $p < 0.05$ significantly higher number of cells infected compared to the number infected
** $p < 0.01$
*** $p < 0.001$ with 8% LS in the adsorbing suspension

‡Samples removed from cell-virus suspensions after 60 minutes adsorption were titrated in duplicate

Table 4.4 Adsorption of virus to BHK-21 cells in suspension: the effect of lamb serum on the efficiency of adsorption

Serum in adsorbing media		pfu/ml of adsorbing suspension‡	% cells infected
Complete	8%	$2.1 \pm 0.49 \times 10^5$	2.1
		$2 \pm 0.48 \times 10^5$	2.0
Complete	4%	$7.2 \pm 1.5 \times 10^{5***}$	7.2
Fraction 1	15%	$3.4 \pm 0.9 \times 10^{5*}$	3.4
		$3.5 \pm 0.79 \times 10^5$	3.5
Fraction 2	15%	$7.8 \pm 1.6 \times 10^{5**}$	7.8
		$6 \pm 1.17 \times 10^5$	6.5
Fraction 3	15%	$1.4 \pm 0.18 \times 10^{6***}$	14.4
		$1.8 \pm 0.18 \times 10^6$	17.5

Input multiplicity 3 pfu/cell, cell concentration 10^7 cells/ml

Adsorption at 37°C for 60 minutes

* $p < 0.05$ significantly higher number of cells infected
** $p < 0.01$ compared to the number infected with 8% LS in the
*** $p < 0.001$ adsorbing suspension

‡Samples removed from cell-virus suspensions after 60 minutes adsorption were titrated in duplicate

Table 4.5 Adsorption of virus to BHK-21 cells: the effect of resuspending cells directly in the virus inoculum

Input multiplicity pfu/cell	Cells/ml in adsorbing suspension	pfu/ml‡ adsorbing suspension	% cells infected
2.5	4×10^7	$4.7 \pm 0.52 \times 10^6$	11.8
		$7.6 \pm 1 \times 10^6$	19.0
		$3.4 \pm 0.1 \times 10^6$	8.5
4.4	3×10^7	$5.9 \pm 0.7 \times 10^6$	19.6
		$7.2 \pm 0.7 \times 10^6$	24.0
		$4.5 \pm 0.4 \times 10^6$	15.0
8.6	2×10^7	$10.8 \pm 1.5 \times 10^6$	54.0
		$11.2 \pm 1.4 \times 10^6$	56.0
		$9.1 \pm 0.3 \times 10^6$	45.5

‡Samples removed from cell-virus suspensions after 60 minutes adsorption were titrated in triplicate

PART II PROPAGATION OF LOUPING-ILL VIRUS IN BHK-21 CELLS

MATERIALS AND METHODS

The maintenance and routine handling of BHK-21 cells was carried out as described in Chapter Two. Unless stated otherwise adsorption of virus was achieved by resuspending trypsinized cells directly in the virus inoculum and incubating at 37°C for 60 minutes. The adsorption procedure and infected cell assay is described in Part I.

Following adsorption of virus, cells were sedimented and washed twice in BHK virus growth media to remove free unadsorbed virus. The cells were resuspended in the appropriate growth media and inoculated into the following culture vessels as indicated below.

1. STATIONARY AND ROLLING TUBE CULTURES

Pyrex glass tubes (15 x 150mm) fitted with rubber stoppers were inoculated with 10^6 cells in a volume of 1.2ml media. The tubes were incubated at 37°C either in stationary racks or on a roller drum rotating at 0.2rpm and were examined microscopically every 24 hours. Ten stationary and 10 rolling tubes were sampled daily for 5 days. The media from each group of 10 was pooled and centrifuged at 750g for 15 minutes at 4°C. The supernatant was stored at -80°C until assayed in duplicate for virus infectivity and HA activity.

2. STATIONARY MULTI-TRAY UNIT*

A multi-tray unit consisting of 10 separate chambers with a total surface area for cell growth of 6000cm^2 was inoculated with 10^9 cells in 1800ml BHK virus growth media. The tray was incubated

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*Inter-Med, Denmark.

at 37°C and the culture harvested after 72 hours incubation. The harvest was stored at -80°C.

3. ROLLING HALF-GALLON BOTTLES*

Half-gallon bottles (length 270mm, diameter 110mm) with a surface area for cell growth of 840cm^2 were each inoculated with 1.5 x 10^8 cells in 200ml BHK virus growth media. The bottles were incubated at 37°C on a roller rotating at 0.2rpm for 72 hours. Growth of virus in serum free media (EBA media) was also examined; following adsorption, bottles were inoculated with 1.5 x 10^8 cells in 200ml BHK cell growth media supplemented with a further 0.1 per cent (w/v) sodium bicarbonate. After 24 hours incubation the media was decanted and the cell monolayer gently washed twice in PBS, 100ml of EBA media was then added and the cultures reincubated for 48 hours. Cultures were then harvested and stored at -80°C.

4. WHEATON ROLLER BOTTLES*

Wheaton roller culture bottles (length 465mm, diameter 110mm) with a surface area for cell growth of 1680cm^2 were each inoculated with 3 x 10^8 cells in 500ml BHK virus growth media. The bottles were incubated at 37°C on a roller rotating at 0.3rpm. Growth of virus in EBA media was also examined as described in 3. Cultures were harvested 72 hours post infection and stored at -80°C.

Harvests were assayed for infectivity using the plaque assay and HA test as described in Chapter Two.

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*Jencons (Scientific) Ltd., Hemel Hempstead.

RESULTS

1. STATIONARY AND ROLLING TUBE CULTURES

Propagation of virus using BHK virus growth media

Trypsinized cells were resuspended in virus inoculum to give an input multiplicity of 8.6 pfu/cell and a cell concentration of 2×10^7 cells/ml. Following 60 minutes adsorption 53 per cent of cells were infected. The adsorbed cells were washed, resuspended in BHK virus growth media and incubated as stationary and rolling cultures.

A cytopathic effect (CPE) was first observed 24-48 hours following infection. As the CPE developed, cells sloughed off and between 72 and 96 hours after infection detachment of cells was almost total.

After 24 hours virus titres in the rolling and stationary cultures reached 3×10^7 pfu/ml and 4.5×10^7 pfu/ml respectively. Levels remained relatively constant for up to 120 hours post-infection. Yields were highest (1.1×10^8 pfu/ml) in stationary cultures incubated for 96 hours (Fig. 4.4). Haemagglutination titres reached maximum (1/128) in rolling cultures 120 hours post-infection and in stationary cultures (1/256) 72 hours post-infection (Fig 4.4). The full results are tabulated in Appendix 4.1.

The effect on viral replication of varying the concentration of lamb serum in the adsorption and growth media

Two aliquots of trypsinized BHK-21 cells were washed and resuspended in BHK virus growth media containing 10 or 5 per cent lamb serum. Five ml of virus inoculum was added to each cell

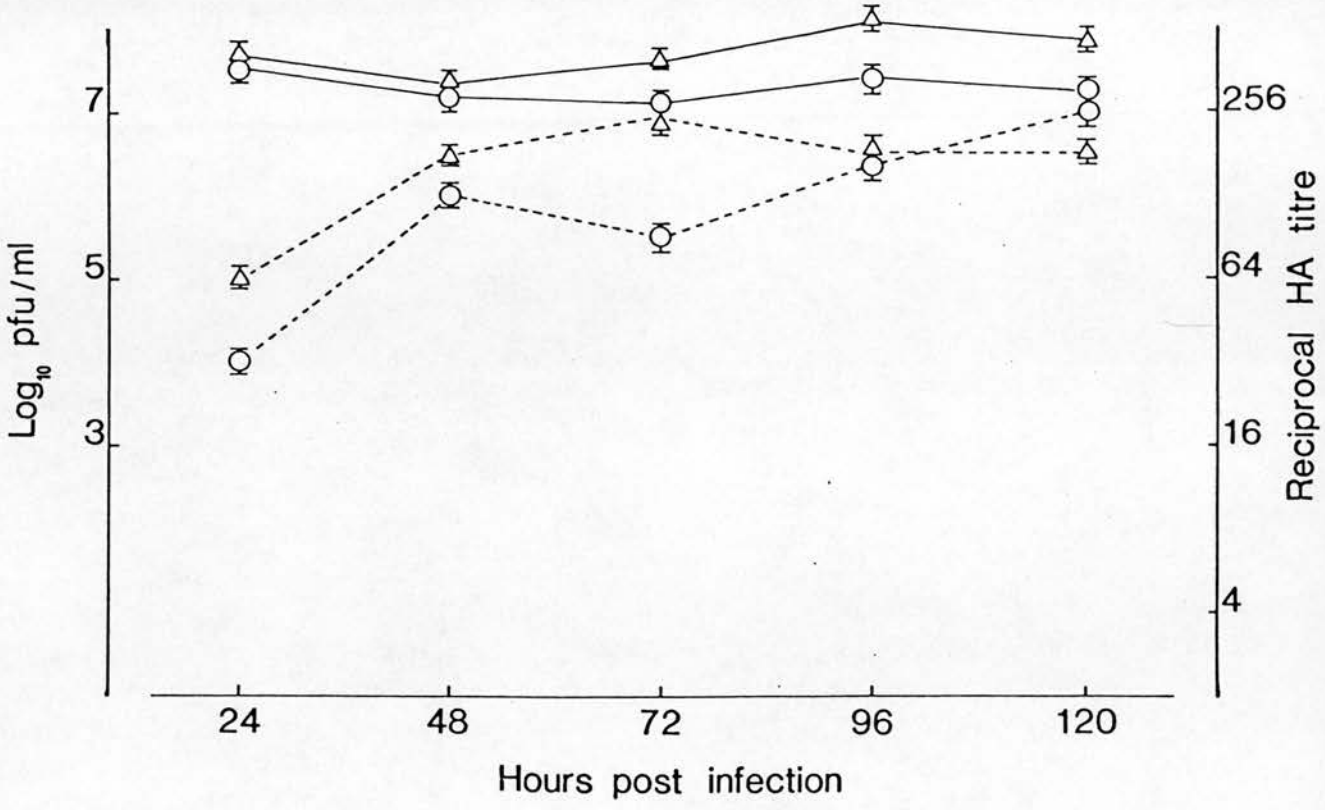
Fig. 4.4 Propagation of louping-ill virus in BHK-21 cells using BHK virus growth media

O——O infectivity titre (log_{10} pfu/ml) in rolling cultures

Δ——Δ infectivity titre (log_{10} pfu/ml) in stationary cultures

O----O reciprocal HA titre in rolling culture

Δ----Δ reciprocal HA titre in stationary culture


Log10 pfu/ml
7
5
3
Reciprocal HA titre
256
64
16
4
24
48
72
96
120
Hours post infection

suspension (10ml) to give an input multiplicity of 1.5 pfu/cell and a cell concentration of 2.2×10^7 cells. Following 60 minutes of adsorption the percentage of infected cells was 7.3 and 10 respectively. The adsorbed cells were washed twice, resuspended in either 10 or 5 per cent lamb serum growth media and incubated either as rolling or stationary cultures.

The highest infectivity titres (1.2×10^8 pfu/ml) were obtained from stationary cultures grown in 10 per cent LS growth media for 48 hours (Fig 4.5). Virus levels were maintained for the period of the experiment. Rolling cultures grown in 10 per cent LS growth media developed maximum titres (4.7×10^7 pfu/ml) after 48 hours incubation, titres then showed a gradual decrease to 1.2×10^6 pfu/ml at 120 hours post-infection. After 24 hours stationary and rolling cultures grown in 5 per cent LS growth media produced virus titres of 2.7×10^7 pfu/ml and 5.2×10^7 pfu/ml respectively, titres however progressively declined and by 120 hours had decreased to 2.8×10^2 pfu/ml and 7.7×10^5 pfu/ml respectively. Virus titres were thus higher in cells cultured in 10 per cent lamb serum.

A similar pattern was observed in HA activity. The highest HA titre (1/512) was obtained from stationary cultures grown in 10 per cent LS media (Fig. 4.6). Titres were lower in stationary and rolling cultures grown in 5 per cent LS media and, after 96 hours incubation had declined to undetectable and minimal levels respectively. The full results are tabulated in Appendix 4.2.

Figure 4.5 The infectivity titre (log_{10} pfu/ml) of louping-ill virus propagated in

O——O rolling BHK-21 cell cultures using BHK virus growth media (10% LS)

Δ——Δ stationary BHK-21 cell cultures using BHK virus growth media (10% LS)

●----● rolling BHK-21 cell cultures using BHK virus growth media (5% LS)

▲----▲ stationary BHK-21 cell cultures using BHK virus growth media (5% LS)

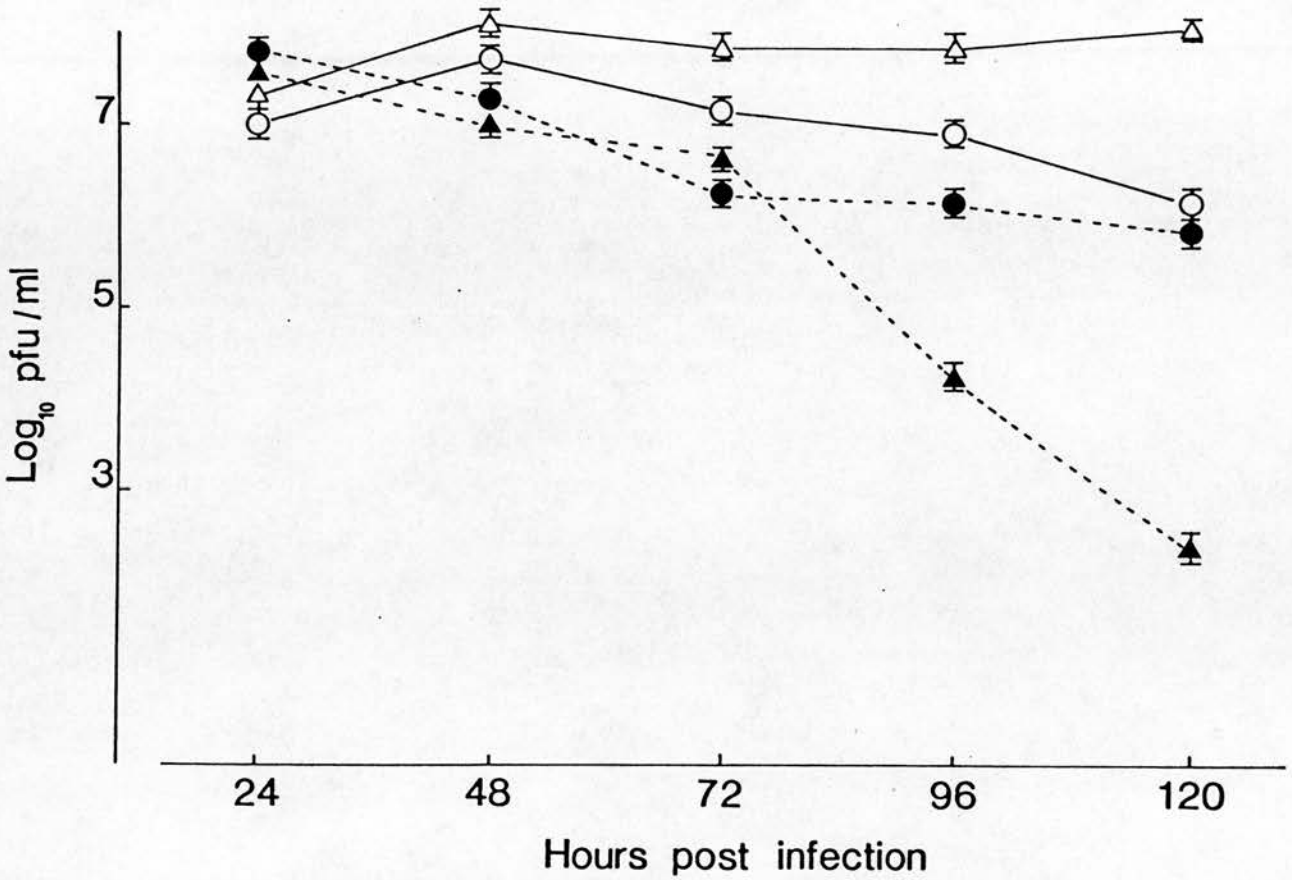

7
5
3
Log10 pfu/ml
24
48
72
96
120
Hours post infection

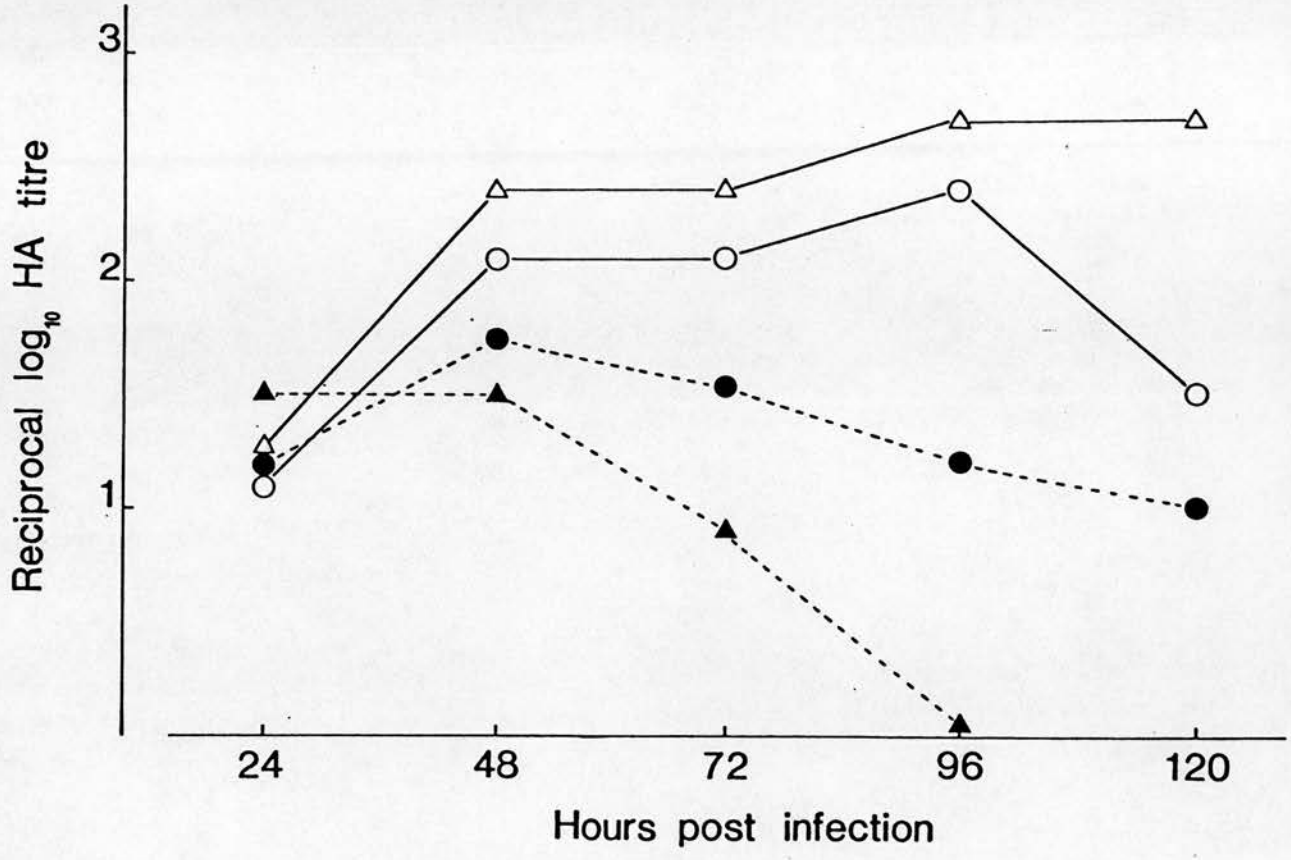
Fig. 4.6 The reciprocal HA titre (log_{10}) of louping-ill virus propagated in

O——O rolling BHK-21 cell cultures using BHK virus growth media (10% LS)

△——△ stationary BHK-21 cell cultures using BHK virus growth media (10% LS)

●----● rolling BHK-21 cell cultures using BHK virus growth media (5% LS)

▲----▲ stationary BHK-21 cell cultures using BHK virus growth media (5% LS)


Reciprocal log10 HA titre
3
2
1
24
48
72
96
120
Hours post infection

Propagation of virus in BHK-21 mycoplasma contaminated cells and BHK-21 mycoplasma-free cells BHK-21 (R8MF)

Trypsinized BHK-21 and BHK-21 (R8MF) cells were washed and each resuspended in 8ml virus inoculum to give an input multiplicity of 1.6 pfu/cell and a cell concentration of 2×10^7 cells/ml. Following 60 minutes adsorption 19 and 8 per cent of BHK-21 and BHK-21 (R8MF) cells respectively were infected. The adsorbed cells were washed twice, resuspended in BHK virus growth media and incubated as rolling and stationary cultures in test tubes. After 48 hours incubation approximately 50 per cent of cells in all cultures appeared rounded and cell detachment was apparent.

Virus titres were consistently higher in BHK-21 (mycoplasma contaminated) cells, incubated both as stationary and rolling cultures. After 96 hours mean virus titres* of 7.9×10^7 pfu/ml and 2.6×10^7 pfu/ml were demonstrated in stationary and rolling cultures of BHK-21 cells compared with 1.3×10^6 pfu/ml and 1.1×10^4 pfu/ml in stationary and rolling cultures of BHK-21 (R8MF) cells (Fig. 4.7). Virus yields remained constant up to 120 hours in all cultures except BHK-21 (R8MF) rolling cell cultures. Virus yields from these cells declined from a maximum titre of 9.1×10^5 pfu/ml 24 hours after infection to a titre of 1.1×10^4 pfu/ml after 120 hours.

The HA activity was shown to parallel the infectivity titres. HA titres were highest (Fig. 4.8) in both stationary and rolling cultures of BHK-21 cells reaching mean titres of 1/512 and 1/427

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*Data collated from two separate experiments carried out under the same adsorption and growth conditions.

Fig. 4.7 The infectivity titre (log_{10} pfu/ml) of louping-ill virus propagated in

O——O rolling cultures of BHK-21 mycoplasma contaminated cells

△——△ stationary cultures of BHK-21 mycoplasma contaminated cells

●----● rolling cultures of BHK-21 mycoplasma-free cells

▲----▲ stationary cultures of BHK-21 mycoplasma-free cells

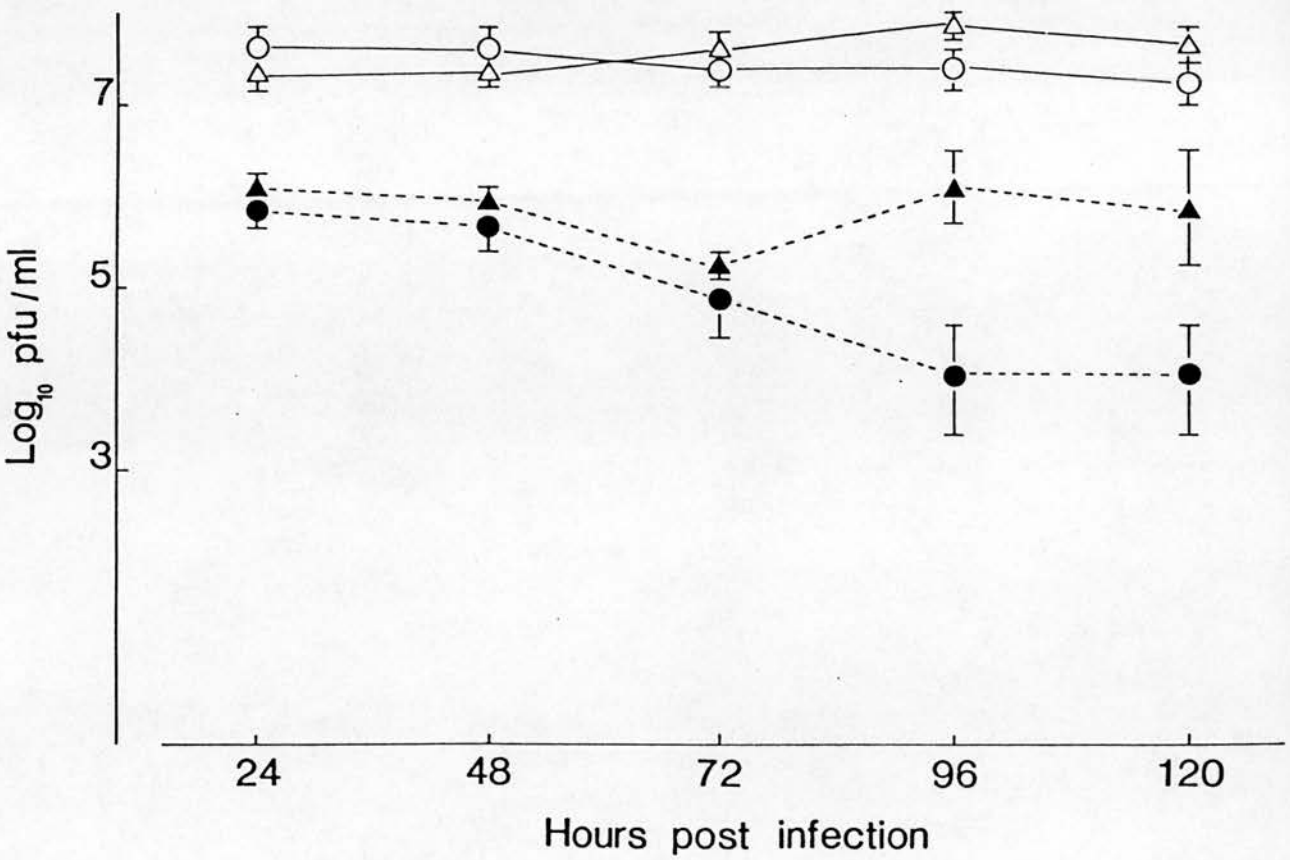

7
5
3
Log10 pfu/ml
24
48
72
96
120
Hours post infection

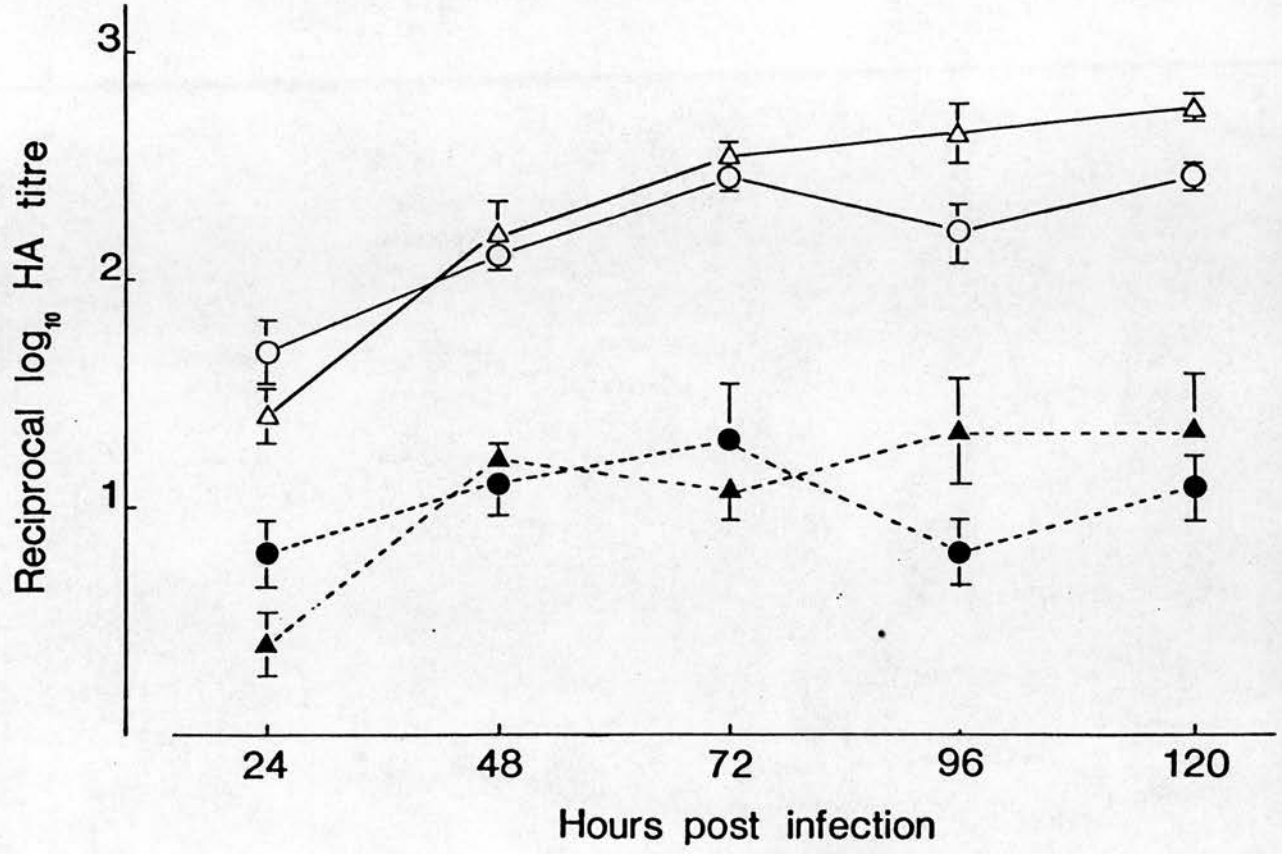
Fig. 4.8 The reciprocal HA titre (log_{10}) of louping-ill virus propagated in

O——O rolling cultures of BHK-21 mycoplasma contaminated cells

△——△ stationary cultures of BHK-21 mycoplasma contaminated cells

●----● rolling cultures of BHK-21 mycoplasma-free cells

▲----▲ stationary cultures of BHK-21 mycoplasma-free cells


Reciprocal log10 HA titre
3
2
1
24
48
72
96
120
Hours post infection

respectively 120 hours post-infection. In contrast a maximum titre of 1/20 was demonstrated in stationary (96 hours p.i.) and rolling (72 hours p.i.) cultures of BHK 21 (R8MF) cells. The full results are shown in Appendices 4.3 and 4.4.

2. STATIONARY MULTI-TRAY UNIT

Trypsinized BHK-21 cells were washed and resuspended in 10ml virus inoculum to give an input multiplicity of 0.5 pfu/cell and a cell concentration of 1.2×10^8 cells/ml. Following adsorption 11 per cent of cells were infected. The adsorbed cells were washed twice, resuspended in BHK virus growth media and cultured in a multi-tray unit. After 72 hours incubation the infectivity of the virus harvest was shown to be 4.5×10^6 pfu/ml and the HA titre 1/16.

3. ROLLING HALF-GALLON BOTTLE CULTURES

Propagation of virus using BHK virus growth media

Trypsinized BHK-21 cells were resuspended in the virus inoculum to give input multiplicities ranging from one pfu/cell to 7.5 pfu/cell. Following adsorption, cells were inoculated into half-gallon bottles containing 200ml BHK virus growth media.

The infectivity titres of the 72-hour-virus harvests ranged from 6×10^7 pfu/ml to 1.1×10^8 pfu/ml with HA titres of 1/128.

Propagation of virus using EBA media

Trypsinized BHK-21 cells were resuspended in the virus inoculum to give input multiplicities of one pfu/cell. The infectivity titres of the 72-hour-virus harvests ranged from 2.9×10^7 pfu/ml to 1.7×10^8 pfu/ml and HA titres were 1/256 and 1/128 respectively.

4. WHEATON ROLLER CULTURE BOTTLES

Propagation of virus using BHK virus growth media

Trypsinized cells were resuspended in virus inoculum to give input multiplicities ranging from 5 to 0.2 pfu/cell.

After 72 hours incubation mean virus titres ranged from 2.8 x 10^7 pfu/ml to 3.5 x 10^8 pfu/ml and mean HA titres were 1/78 to 1/355. The full results are tabulated in Appendix 4.5.

Propagation of virus using EBA media

The infectivity titres of the 72-hour-virus harvests ranged from 1 x 10^8 pfu/ml to 2 x 10^8 pfu/ml and HA titres were 1/512 and 1/256.

DISCUSSION

Edward (1948a) proposed that for a formalinized vaccine prepared from the virus infected tissues of one species to be effective when inoculated in another species a sufficiently high concentration of antigen must be present. Louping-ill vaccine, required primarily for the immunization of sheep and derived from BHK-21 cells thus presents such a heterologous system. The necessity therefore for optimizing the growth of louping-ill virus in BHK-21 cells to consistently produce harvests of maximum infectivity is a prerequisite in subsequent studies on vaccine preparation and potency.

Louping-ill virus was shown to propagate to maximum titres, $>10^8$ pfu/ml in BHK-21 mycoplasma contaminated cells incubated both as stationary and rolling cultures in BHK virus growth media or EBA media. Maximum titres (mouse LD_{50}/0.03ml) of louping-ill virus

propagated in other systems for vaccine production have previously been shown to be $10^{-5.1}$ in sheep kidney cells (Brotherston and Boyce, 1970), 10^{-8} in infected mouse brain (Edward, 1948b), $10^{-7.5}$ in infected chick embryo (Edward, 1947a) and 10^{-5} in infected sheep tissue (Gordon, Brownlee, Wilson and MacLeod, 1962).

The titres of virus achieved in these studies are therefore greater than any previously described following propagation of virus in vivo or in vitro.

Fluorescent studies indicated that specific antigen was first apparent 10-12 hours after infection of cells and was localized around the cell nucleus. By 24 hours post-infection fluorescence was observed throughout the cytoplasm of all cells. Libíková and Albrecht (1962) studying the replication of tick-borne encephalitis (TBE) virus in HeLa cell monolayers similarly observed fluorescent antigen present in the perinuclear area between 8 and 11 hours post-infection; by 17 hours fluorescence was apparent throughout the cytoplasm of all cells.

Final yields of louping-ill virus, harvested 72 hours post-infection were similar regardless of the input multiplicity; Mayer, Zemla and Albrecht (1962) likewise demonstrated that final yields of TBE virus propagated in HeLa cells and harvested at 72 hours were of the same order even though the input multiplicity was varied.

The infectivity titres of virus grown in media containing 10 per cent lamb serum were similar to titres of virus grown in EBA media. Under the same conditions however the infectivity of harvests grown in media containing 5 per cent lamb serum were lower.

BHK-21 clone 13 cells routinely used have been shown to be contaminated with Mycoplasma arginini and other unidentified mycoplasmas of human and ovine origin (G. Jones, personal communication). Mycoplasma contamination of cell cultures has been shown to influence virus growth producing either a decrease or increase in virus yields. Rouse, Bonifas and Schlesinger (1963) and Singer, Fitzgerald, Barile and Kirschstein (1970) demonstrated that mycoplasmas decreased the yields of adenovirus type 3 and vaccinia virus respectively by depleting arginine from the growth media. Singer, Barile and Kirschstein (1969) studying the effect of mycoplasma infection on the production of interferon and the effect on yields of Semliki Forest virus in hamster embryonic kidney cells demonstrated that M. arginini and M. hyorhinis inhibited interferon production resulting in increased yields of virus. The enhancement of virus production was however only demonstrated when cells were infected with a low input of virus and it was postulated that with a low virus input only a small number of cells are initially infected leaving most of the cells susceptible to interferon. In cells contaminated with M. arginini and M. hyorhinis interferon production was inhibited and uninfected cells remained susceptible to virus infection and yields were high. In mycoplasma-free cells interferon production was not inhibited and with a low virus input the remaining uninfected and interferon-sensitive cells developed a degree of resistance to virus infection and virus yields were low. With a high virus input the number of cells infected initially was substantially increased and the effect of mycoplasma inhibition of interferon production was thus masked.

Propagation of louping-ill virus in BHK-21 mycoplasma-contaminated cells resulted in virus titres 2.3 $\log_{10}$ pfu/ml higher than titres of virus grown in BHK-21 (R8MF) mycoplasma-free cells. The virus input was one pfu/cell. Whether the enhancing effect on virus growth by contaminating mycoplasmas involves interferon and is thus analogous to the effect described by Singer, Barile and Kirschstein (1969) is unknown and a more detailed study and a better understanding of the relationship between virus, cell and mycoplasma is required.

For commercial production it was found that yields of virus propagated under the optimal conditions described reached maximal titres, $>10^8$ pfu/cell, in both Wheaton roller culture bottles and half-gallon roller bottles. Yields of virus were considerably lower from cells grown stationary in a multilayer plate. Production of the present commercial vaccine utilizes Wheaton roller culture bottles for propagation of virus in batches up to 40 litres. The obvious advantage of this culture bottle as opposed to the half-gallon bottle is the increased surface area for cell growth. Propagation of virus in BHK-21 cells adapted to grow in suspension was not examined, although it is recognised that such an investigation would be particularly pertinent to the need for producing large volumes of high titred virus for vaccine production.

CHAPTER FIVE

THE INACTIVATION OF LOUPING-ILL VIRUS BY TREATMENT WITH FORMALIN

INTRODUCTION

An inactivated louping-ill vaccine suitable for commercial exploitation was first developed by Gordon (1934) using infected sheep brain, spinal cord and spleen tissues treated with 0.35 per cent formalin (final concentration) for 4 days at 0-5°C. In a subsequently developed tissue culture-derived louping-ill vaccine (Brotherston and Boyce, 1970) virus harvests were inactivated by exposure to 0.1 per cent formalin (final concentration) for 24 hours at 37°C. This latter method of inactivation is also employed to inactivate virus propagated in BHK-21 cells during commercial production of the presently available inactivated louping-ill vaccine.

The process of inactivation of louping-ill virus by formalin has not however been studied in detail and this chapter therefore examines the inactivation of tissue culture-derived louping-ill virus, describing the dynamics of the inactivation process and the effect on the rate of inactivation of using lower concentrations of formalin. The selection of the optimal concentration of formalin with regard to subsequent vaccine manufacture is also discussed.

MATERIALS AND METHODS

Preparation of virus

Louping-ill virus was propagated in BHK-21 cells grown in BHK virus growth media and incubated at 37°C as rolling cultures. After

72 hours incubation the infected supernatants were harvested, samples removed for infectivity assay and the remainder dispensed in 15ml volumes in screw-capped bottles.

Virus inactivation

The course of virus inactivation in tissue culture fluids was followed at 37°C with final formalin* dilutions of 1/1000, 1/2000, 1/4000 and 1/8000. The fluids were kept in screw-capped bottles in a 37° ± 0.5°C incubator. Virus to which no formalin had been added was also incubated at 37°C to determine the lability of louping-ill virus at this temperature. Aliquots were removed from the preparations immediately following the addition of formalin and thereafter at various time intervals and assayed for residual virus and haemagglutination activity. Deformalinization of aliquots was attempted using a 2.3 per cent aqueous solution of $NaHSO_3$ in the ratio 1mol $NaHSO_3$ per 1mol of formaldehyde. It was shown however that treatment of live virus with the highest concentration of $NaHSO_3$ decreased infectivity by 1.5 $\log_{10}$ pfu/ml and HA activity to undetectable levels. Inactivated samples were therefore not deformalinized.

Residual virus assay

Immediately following sampling aliquots were diluted in PK/BA media and assayed for residual virus using the plaque method described in Chapter Two.

Haemagglutination assay

Aliquots (0.4ml volume) were dispensed into screw-capped bottles

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*Analar Formaldehyde solution (37-40%) BDH Chemicals Ltd.

and stored at -20°C until completion of the experiment. All samples were then assayed for haemagglutination activity using the method described in Chapter Two.

RESULTS

The course of formalin inactivation of louping-ill virus in tissue culture fluid is illustrated in Fig. 5.1. The curves obtained show that there was an initial rapid drop in infectivity in the four hours immediately following addition of formalin. With formalin dilutions of 1/1000 and 1/2000 infective virus was not detected after 12 hours incubation at 37°C. The rate of inactivation with formalin at 1/4000 and 1/8000 was slower and the time required for complete inactivation of virus was protracted to 24 and 48 hours respectively.

Haemagglutination activity was not detected after 4 hours and 12 hours exposure of virus to formalin dilutions of 1/1000 and 1/2000 respectively. With formalin dilutions of 1/4000 and 1/8000 HA activity was detectable up until 24 hours and 48 hours of incubation with corresponding titres of 1/8 and 1/32.

Incubation of virus without formalin at 37°C for 12 hours caused no appreciable loss in infectivity, after 24 and 48 hours infectivity was reduced by 1.0 log and 3.0 logs respectively. Haemagglutination activity with a titre of 1/128 was maintained throughout the sampling period.

DISCUSSION

Inactivation of louping-ill virus in tissue culture fluids with 1/1000 and 1/2000 formalin resulted in a loss in detectable

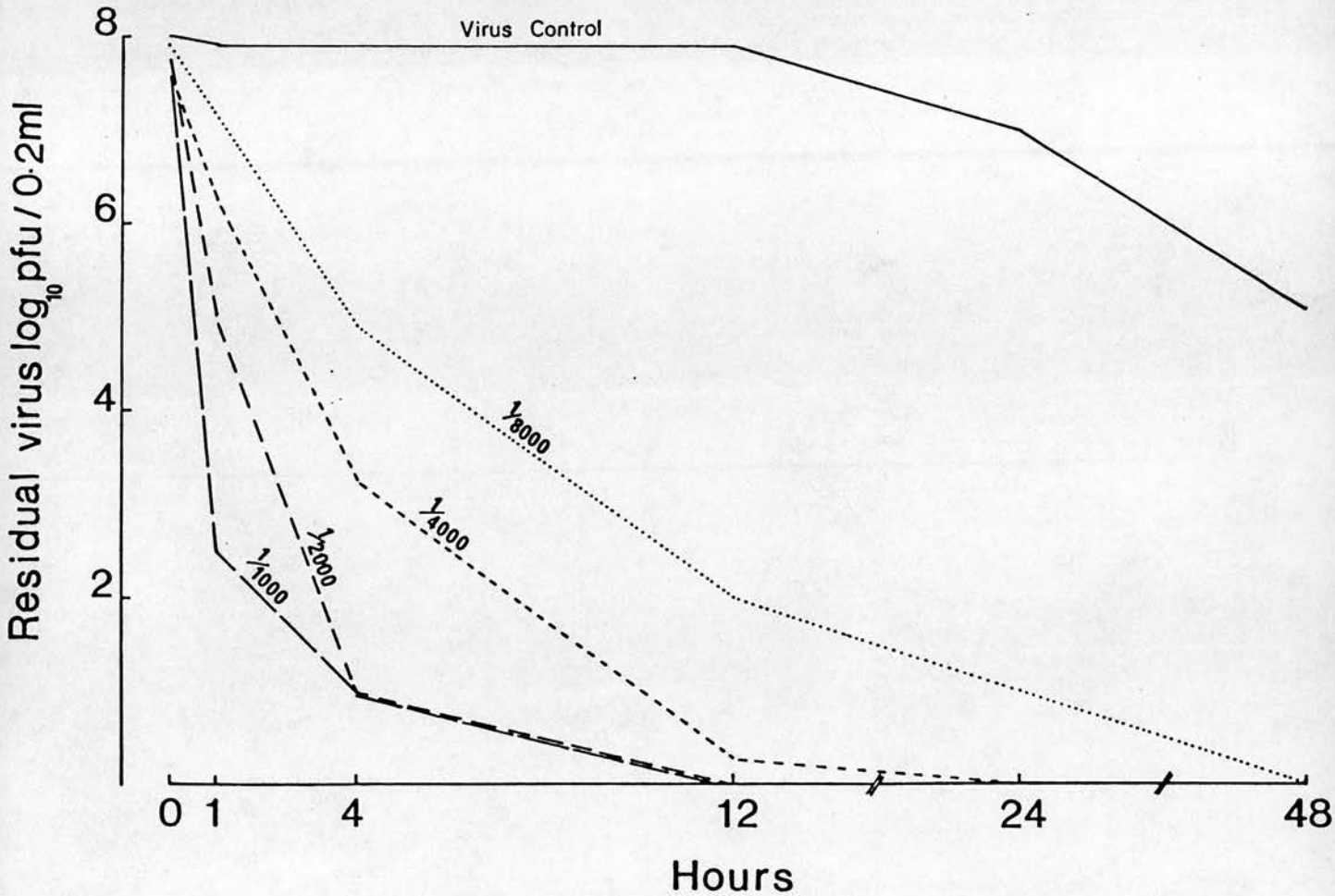
Fig. 5.1 The course of formalin inactivation of louping-ill virus propagated in BHK-21 cells in BHK virus growth media

Formalin concentrations: 1/8000 .....

1/4000 – – –

1/2000 —— ——

1/1000 —·—— ——


Virus Control
1/8000
1/4000
1/2000
1/1000
Residual virus log10 pfu/0·2ml
8
6
4
2
0 1 4 12 24 48
Hours

infectivity after 12 hours incubation at 37°C. The curve representing the rate of the formalin inactivation reaction was not a straight line but biphasic, indicating a higher rate of inactivation in the first 4 hours following addition of formalin. A similar biphasic reaction was observed in the inactivation of Japanese B encephalitis virus with formalin dilutions ranging from 1/1000 to 1/8000 (Darwish and Hammon, 1966a) and also in inactivation of poliovirus exposed to a formalin concentration of 1/8000 (Timm, MacLean, Kupsky and Hook 1956). Charney, Fisher and Machlowitz (1957) studying the inactivation of a purified poliovirus suspension obtained however straight line inactivation curves indicating a reaction following first order kinetics.

The lability of louping-ill virus to formalin is characteristic of a number of togaviruses. Bartelloni, McKinney, Calia, Ramsburg and Cole (1971) and Maire, McKinney and Cole, (1970) demonstrated that tissue culture grown Western equine encephalitis virus and Venezuelan equine encephalitis virus were inactivated by 1/2000 formalin at 37°C in less than 24 hours. Danes and Benda (1960) observed that tick-borne encephalitis virus strain B3 incubated at 4°C for 72 hours was inactivated by formalin at a dilution of 1/2000, and Gall (1973) studying the inactivation of tick-borne encephalitis virus strain B7 showed that incubation with 1/4000 formalin for 12 hours at 37°C resulted in loss of detectable infectivity.

The formalin dilution required in the preparation of an inactivated vaccine is determined by the need to completely destroy infectivity yet maintain antigenicity. High concentrations of

formalin, although causing a rapid loss of infectivity, have been shown to cause physical damage of virus particles (Schaffer, 1960) and antigenicity of the inactivated virus suspension may be impaired. Salk and Gori (1960) have however shown that when very low concentrations of formalin are used most of the formalin will only bind with the protein surface layer with minimal diffusion to the interior of the virus particle and this may result in a reversible type of virus inactivation. In addition low concentrations of formalin also prolong the incubation period required for complete destruction of infectivity and the antigenicity of preparations inactivated at 30°C or 37°C may be adversely affected as a result of thermal denaturation of the virus particle (Salk and Gori, 1960). Inactivation at lower temperatures although abrogating thermal denaturation of virus greatly decreases the rate of inactivation (Darwish and Hammon, 1966c; Robinson, Berman, Lowenthal and Hetrick, 1966; White, Berman and Lowenthal, 1971), and the period of incubation required for complete loss of infectivity may be impractical for a vaccine production schedule. Cole, May and Robinson (1973) have however shown that the immunogenicity of inactivated Venezuelan equine encephalitis vaccines remained stable even after treatment with 0.1 per cent formalin for 96 hours at 37°C and Danes and Benda (1960) observed no significant differences in the immunogenicity of tick-borne encephalitis vaccines inactivated by formalin dilutions ranging from 1/500 to 1/2000 or in the immunogenicity of formalinized and the corresponding deformalinized vaccines.

A formalin dilution that is mild yet effective and can be tolerated in a vaccine without the necessity for subsequent

elimination by dialysis or chemical means is therefore required. A formalin dilution of 1/2000 was shown to completely destroy detectable louping-ill virus infectivity within 24 hours and is therefore as efficient as a dilution of 1/1000. Treatment with the 1/2000 dilution however constitutes a milder yet equally functional inactivation process. Higher dilutions of formalin were shown to require longer incubation periods to completely inactivate virus and this may adversely affect antigenicity. It is suggested therefore that for preparation of inactivated louping-ill virus vaccine a formalin dilution of 1/2000 is optimum.

CHAPTER SIX

THE IMMUNOGENICITY OF METHANOL-CONCENTRATED AND UNCONCENTRATED LOUPING-ILL VIRUS VACCINES

INTRODUCTION

Proteins are readily precipitated out of solution by treatment with organic solvents, and the use of methanol to precipitate protein from virus infected tissue culture fluids is well established (Harris, 1964). Methanol precipitation of virus harvests and subsequent elution of viral protein from non-viral protein without a significant loss in infectivity has been described with influenza virus (Cox, Van der Scheer, Aiston and Bohnel, 1947), Eastern equine encephalitis virus, influenza B virus and Newcastle disease virus (Pollard, Connolly and Fromm, 1949) and poliomyelitis virus (Schwerdt and Schaffer, 1956), resulting in concentrated and partially purified virus preparations.

Concentration of louping-ill virus (propagated in secondary monolayers of sheep kidney cells) by methanol or acetone precipitation was shown to produce immunogenic vaccines (Brotherston and Boyce, 1970). High antibody titres were regularly induced by a single dose of such vaccines. No attempts were made to elute virus from the non-viral protein present, rather, the entire protein precipitate was used as antigen. Vaccines prepared from unconcentrated harvests were shown to be non-immunogenic even following the administration of 2 doses.

For commercial production of louping-ill virus vaccine, virus is propagated in BHK-21 cell monolayers and virus antigen concentrated by methanol precipitation (Appendix 3.1). The concentration procedure employed is essentially that described by Brotherston and Boyce (1970). Experience has indicated however that the potency of the commercially produced vaccine is variable and sometimes considerably lower than the experimental louping-ill vaccine described by Brotherston and Boyce (1970). Two doses of commercial vaccine are required to consistently provoke a satisfactory immune response.

The potency of inactivated vaccines has been shown to be dependent on the infectivity of the source material (Smorodintsev and Ilyenko, 1962; White, Berman and Lowenthal, 1972; Levkovich, 1962; Crawford, Dayhuff and White, 1968; Edward, 1948b and Cox, Van der Scheer, Aiston and Bohnel, 1947). The relationship between the immunogenicity of louping-ill vaccines and the infectivity titre of the virus harvest prior to inactivation, and an evaluation of methanol precipitation as a means of concentrating commercially produced louping-ill virus are described in this chapter. The requirement for an effective method of concentrating virus to consistently produce vaccines of satisfactory potency is also discussed.

MATERIALS AND METHODS

1. THE EFFICACY OF METHANOL TREATMENT FOR CONCENTRATING VIRAL ANTIGEN: A COMPARISON OF THE IMMUNOGENICITY OF VACCINES PREPARED FROM CONCENTRATED VIRUS HARVESTS AND UNCONCENTRATED VIRUS HARVESTS.

All the vaccines used in this study were derived from inactivated, concentrated or unconcentrated tissue culture harvest pools prepared during the commercial production of louping-ill virus vaccine in 1978 and 1979. Inactivation of the harvests and concentration by methanol precipitation is described in Appendix 3.1.

The data concerning the immunogenicity of vaccines prepared from the concentrated virus harvest pools were derived from the pre-blend potency assays described in Appendix 3.1. Essentially, aliquots of the 10-fold concentrated virus harvest pools (A-F) were diluted to give 5-, 2.5- and 1.25-fold concentrates using PBS plus 0.5 per cent (v/v) phenol. Vaccines were prepared by emulsifying each of the concentrates with Bayol F and Falba oil adjuvant in a ratio of one part of concentrate to 2 parts adjuvant to give a final volume of 15ml. Emulsification was carried out using a MSE bench type overhead homogenizer. The vaccines were prepared and assayed for potency immediately following production of each concentrated virus harvest pool.

Vaccines were also prepared from aliquots of the corresponding unconcentrated inactivated virus harvest pools (A-F). Prior to inactivation, vaccine preparation and potency assay the infected virus harvest pools had been stored at -20^{o}C for approximately 5 months.

Various breeds of sheep born either at the Moredun Research Institute or in other areas free of ticks and aged between 7 months and 2 years were used to assess vaccine potency. Prior to vaccination all the sheep were tested for the presence of louping-ill HAI serum antibodies and all were found to be seronegative. Each vaccine was injected sub-cutaneously into 5 sheep behind the right shoulder in a volume of 1ml. All the sheep were then bled weekly for 4 weeks. Twenty-eight days following vaccination sheep inoculated with vaccines prepared from concentrated and unconcentrated virus harvest pools E and F were re-injected with a second 1 ml dose of vaccine. These sheep were then bled weekly for a further 4 weeks. Sheep inoculated with vaccines prepared from unconcentrated virus harvest pools were also bled 10 and 16 weeks post vaccination. The blood was allowed to clot and the sera removed and tested for HAI antibody to louping-ill virus as described in Chapter Two.

Statistical analysis

Reciprocal HAI titres obtained at 7, 14, 21 and 28 days post-vaccination were converted to $\log_{10}$ values and the mean titres calculated. The responses to vaccines prepared from the 6 concentrated virus harvest pools and concentrate dilutions were compared with the responses to vaccines prepared from each of the corresponding unconcentrated virus harvest pools. The data from all vaccines was composited and similarly compared. Tests used to analyse the data included (in order of preference) Dunnett's Multiple Comparison test (Dunnett, 1955); the 2-sample t-test, the Mann Whitney non-parametric test and the Chi-square test.

2. IMMUNOGENICITY OF VACCINES PREPARED FROM AN UNCONCENTRATED VIRUS HARVEST E2

An aliquot of an inactivated and unconcentrated virus harvest, E2, prepared during the commercial production of vaccine was obtained. The infectivity of the harvest prior to inactivation was 3.4×10^8 pfu/ml. The inactivated harvest was diluted 1/3, 1/6, 1/12 and 1/24 using PBS plus 0.5 per cent (v/v) phenol, vaccines were prepared from the undiluted harvest and the diluted preparations as described earlier. Each vaccine was injected sub-cutaneously in a 1ml volume into each of 5 7-month-old seronegative Dorset ewe lambs. The lambs were then bled weekly for 4 weeks and then again at 8 and 12 weeks. The blood was allowed to clot, the sera removed and tested for HAI antibody activity.

RESULTS

1. THE IMMUNOGENICITY OF VACCINES PREPARED FROM CONCENTRATED VIRUS HARVESTS AND THEIR CORRESPONDING UNCONCENTRATED HARVESTS

The 14 and 28 day antibody responses to vaccines prepared from the 10-, 5-, 2.5- and 1.25-fold concentrates of each of the harvest pools (A-D) and to vaccines prepared from the corresponding unconcentrated harvest pools are illustrated in Figs. 6.1 a-d. The antibody responses following revaccination with vaccines prepared from concentrated and unconcentrated virus harvest pools E and F are illustrated in Figs. 6.1e and 6.1f. The 28-day mean reciprocal HAI titres ($\log_{10}$) and the statistical differences in HAI responses elicited by the concentrated and the corresponding unconcentrated vaccines are indicated in Table 6.1. The infectivity titres of the

Fig. 6.1 a-d The 14 and 28 day antibody responses to vaccines prepared from harvest pools A-D

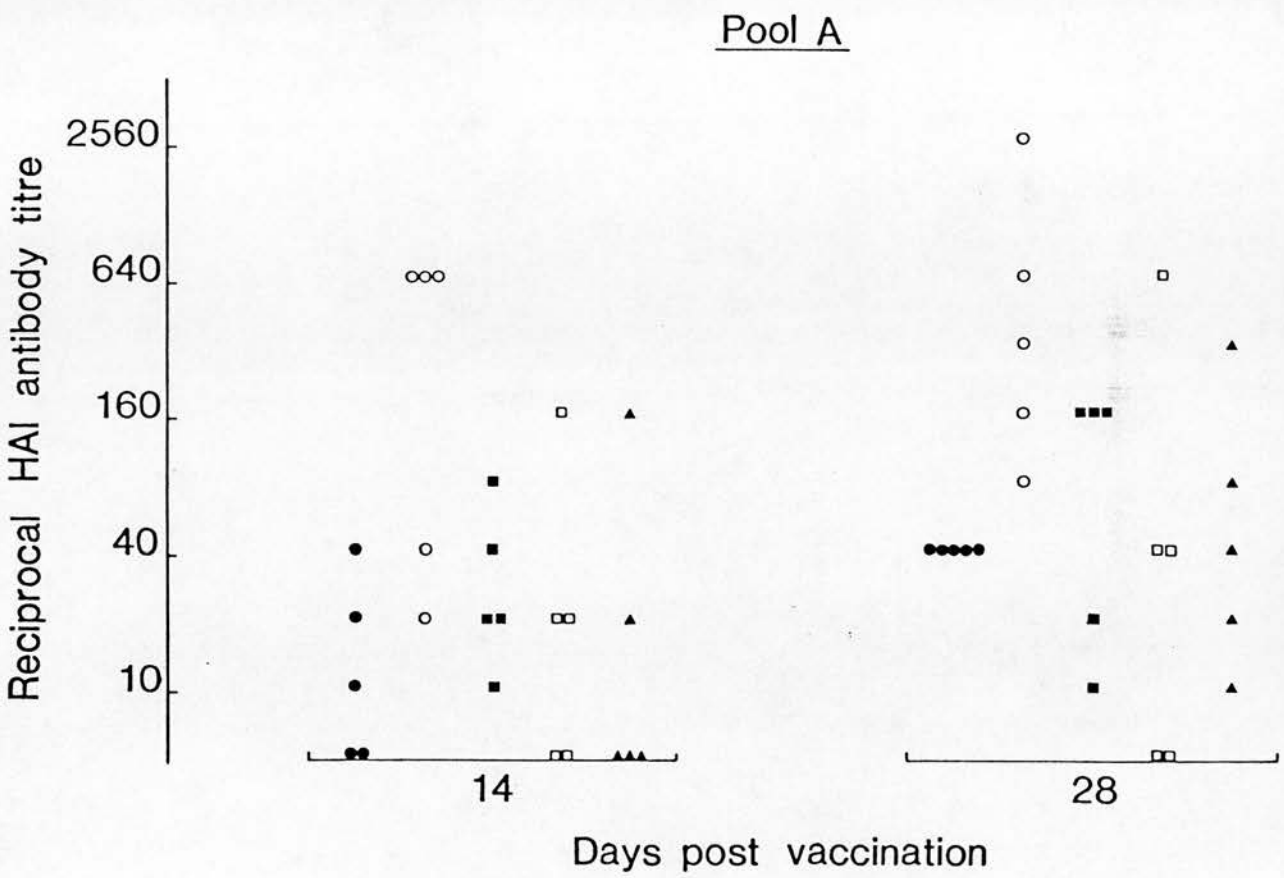
● unconcentrated vaccine

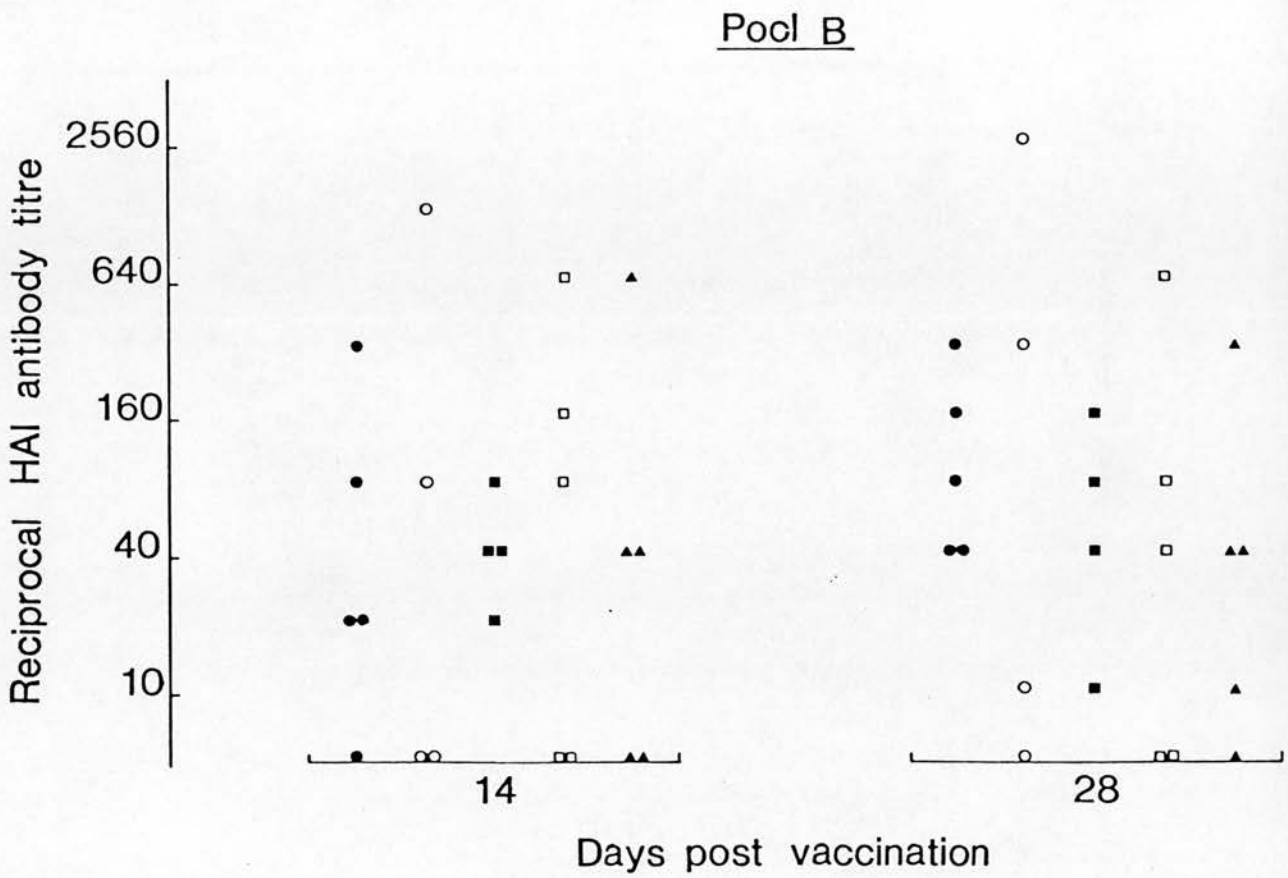
○ 10-fold concentrate vaccine

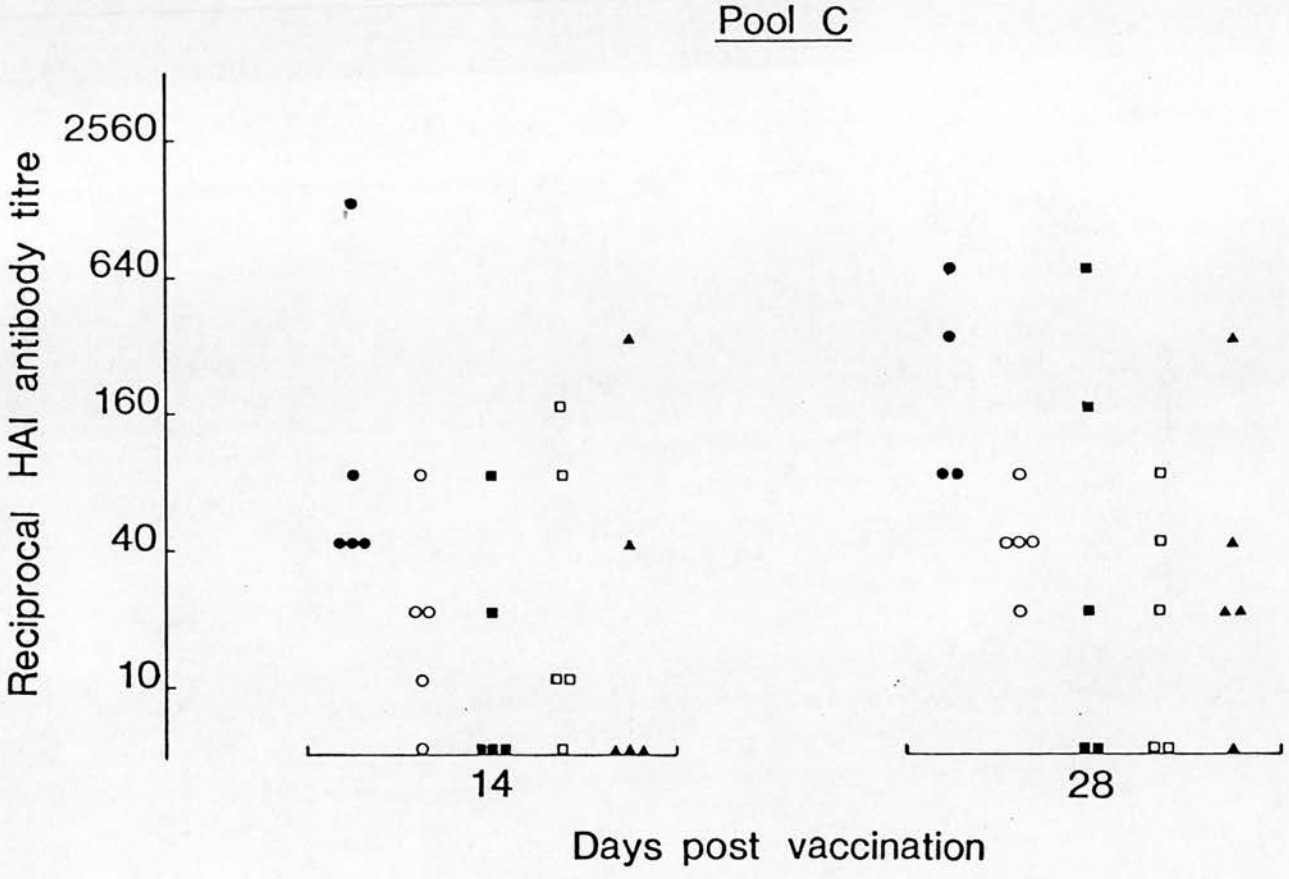
■ 5-fold concentrate vaccine

□ 2.5-fold concentrate vaccine

▲ 1.25-fold concentrate vaccine


Pool A
Reciprocal HAI antibody titre
2560
640
160
40
10
14
28
Days post vaccination


Pool B
Reciprocal HAI antibody titre
2560
640
160
40
10
14
28
Days post vaccination


Pool C
Reciprocal HAI antibody titre
2560
640
160
40
10
14
28
Days post vaccination

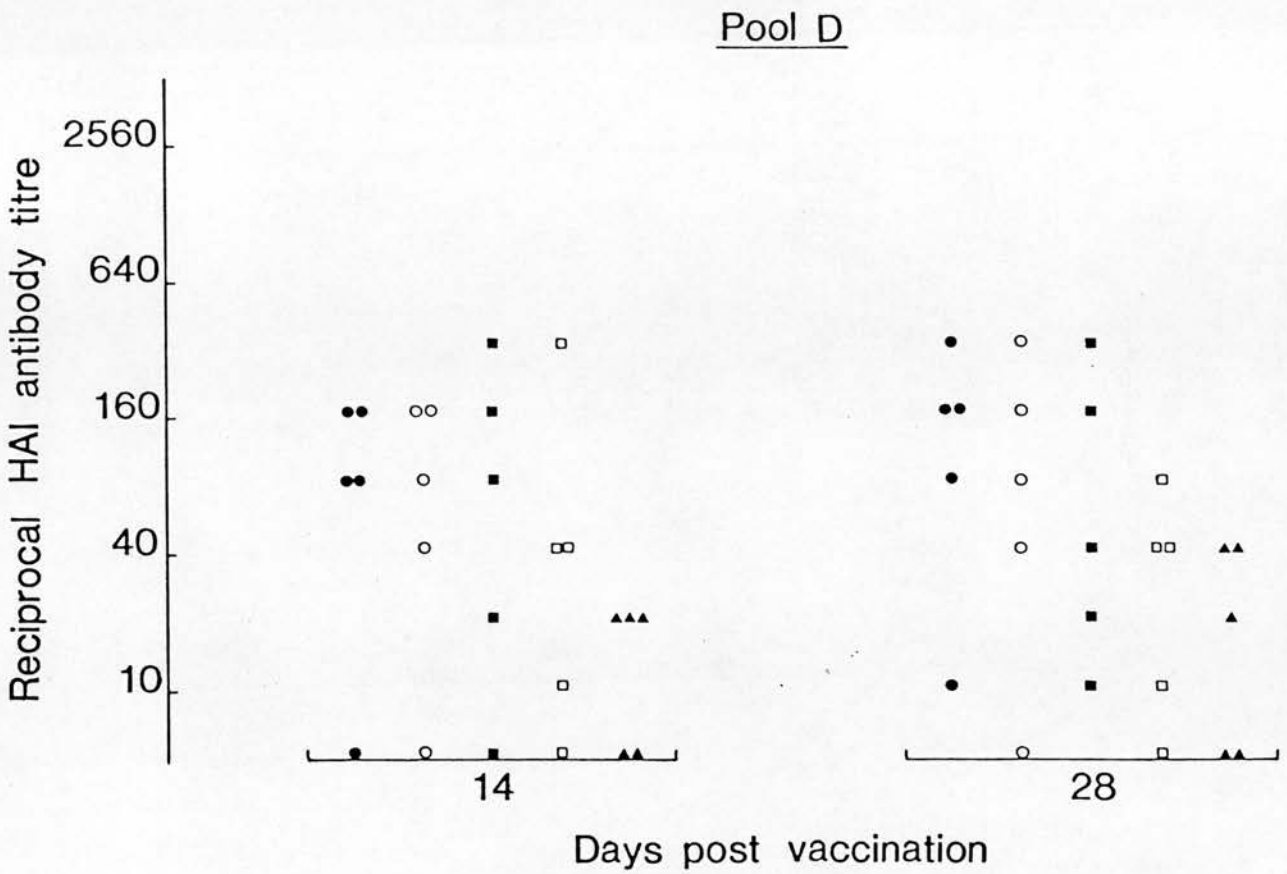

Pool D
Reciprocal HAI antibody titre
2560
640
160
40
10
14
28
Days post vaccination

Fig. 6.1 e-f The 28 and 56 day antibody responses to vaccines prepared from harvest pools E and F

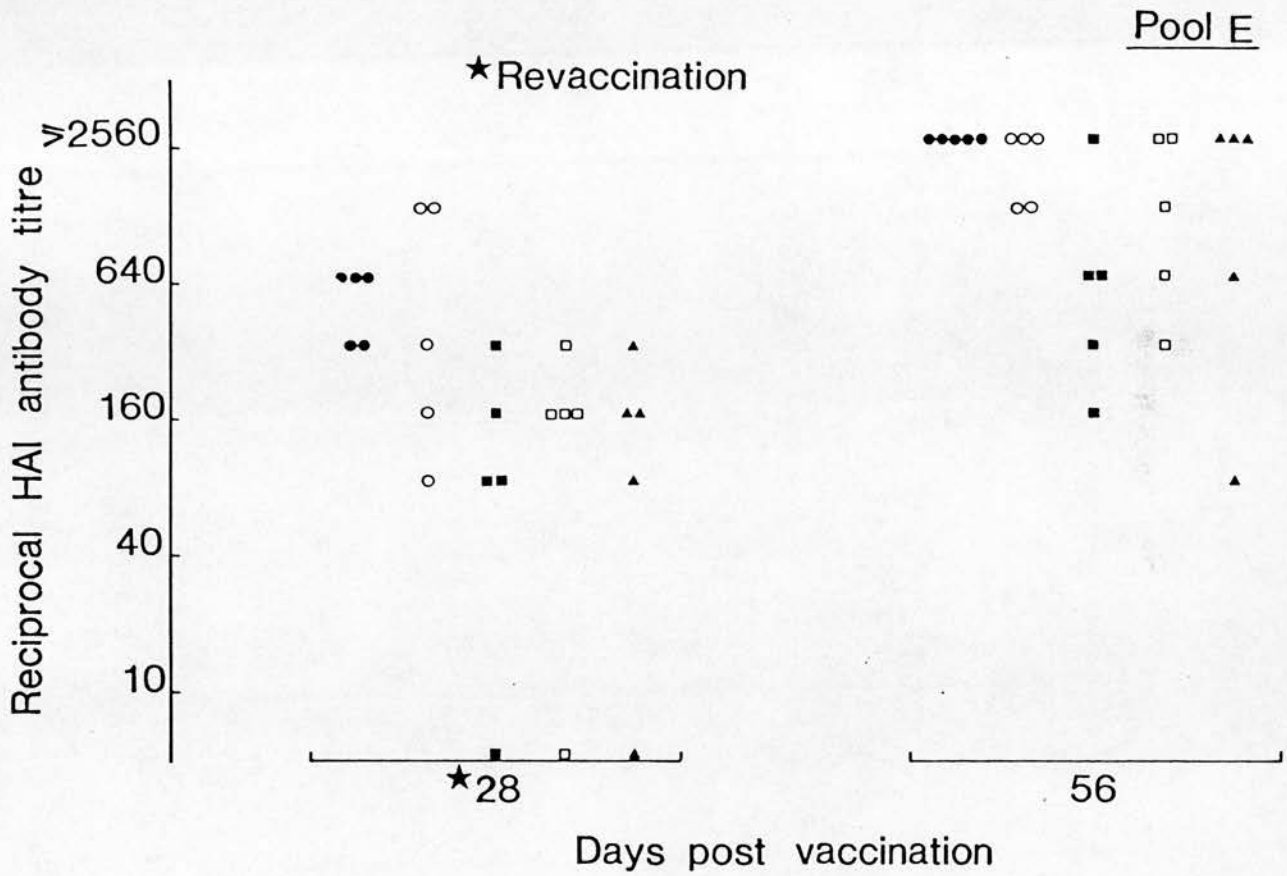
● unconcentrated vaccine

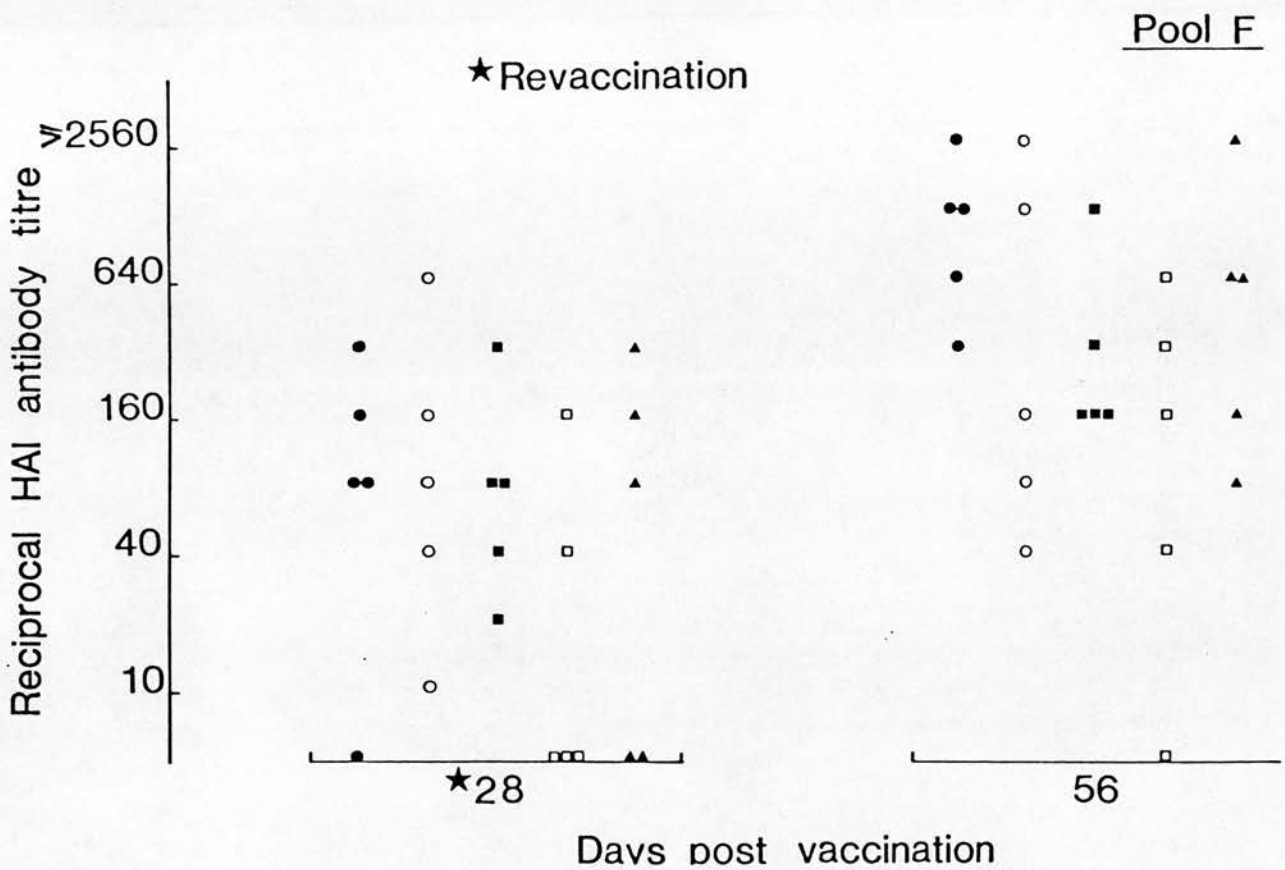
○ 10-fold concentrate vaccine

■ 5-fold concentrate vaccine

□ 2.5-fold concentrate vaccine

▲ 1.25-fold concentrate vaccine


Pool E
★Revaccination
Reciprocal HAI antibody titre
≥2560
640
160
40
10
★28
56
Days post vaccination


Pool F
★Revaccination
Reciprocal HAI antibody titre
≥2560
640
160
40
10
★28
56
Days post vaccination

virus harvests (A-F) prior to inactivation ranged from 8.4 $\log_{10}$ pfu/ml to 8.1 $\log_{10}$ pfu/ml (Table 6.1). The composite mean reciprocal HAI titres ($\log_{10}$) at 14, 21 and 28 days and the statistical differences in the overall responses are tabulated in Table 6.2. The full results are given in Appendix 6.1.

Twenty-eight days after vaccination only one out of the 24 concentrated vaccines produced a significantly higher ($p<0.01$) response than the corresponding unconcentrated vaccine, this vaccine had been prepared from Pool A 10-fold concentrate. Twenty-two of the 24 concentrated vaccines evoked responses that were not significantly different compared to those produced by the unconcentrated vaccines, and, vaccine prepared from Pool C 2.5-fold concentrate produced a significantly lower antibody response ($p < 0.05$) than the corresponding unconcentrated vaccine. The percentage of animals injected with the unconcentrated vaccines or the concentrated vaccines and found to be persistently seronegative for up to 28 days post-vaccination was 3.4 and 19.4 per cent respectively. The highest mean HAI titre (1/501) was detected in animals injected with unconcentrated vaccine (harvest pool E) 28 days following vaccination.

Statistical analysis of the composite data indicated that, overall, 7, 14, 21 and 28 days post-vaccination there was no significant difference between the antibody response induced by the 10- and 5-fold concentrated vaccines and the unconcentrated vaccines. At 21 and 28 days following vaccination the overall response produced by unconcentrated vaccine was significantly higher ($p < 0.001$, $p < 0.01$) than that produced by 2.5-fold concentrate vaccines and that produced by 1.25-fold concentrate vaccines ($p < 0.001$, $P < 0.05$) (Table 6.2).

The immune responses produced by vaccines prepared from unconcentrated harvests were maintained for up to 16 weeks post-vaccination (Table 6.3) and a typical secondary anamnestic response was detected in sheep revaccinated either with unconcentrated or concentrated vaccine (Figs. 6.1 e-f).

2. THE IMMUNOGENICITY OF VACCINES PREPARED FROM UNCONCENTRATED VIRUS HARVEST E2

The infectivity titre of harvest E2 and the equivalent infectivity titres of the 1/3, 1/6, 1/12 and 1/24 diluted preparations were 3.4×10^8 pfu/ml, 1.13×10^8 pfu/ml, 5.6×10^7 pfu/ml, 2.8×10^7 pfu/ml and 1.4×10^7 pfu/ml respectively.

The 14- and 28-day serological responses to vaccines prepared from harvest E2 and harvest dilutions are illustrated in Fig. 6.2. The mean reciprocal HAI ($\log_{10}$) titres 7, 14, 21, 28, 56 and 84 days post-vaccination and the numbers of sheep responding to vaccination are indicated in Table 6.4.

The highest mean HAI titre (1/200) was detected at 21 days in sheep injected either with vaccine prepared from the undiluted harvest or vaccine prepared from the 1/3 harvest dilution, and at 28 days mean HAI titres were shown to be 1/200 and 1/100 respectively. In addition a serological response was demonstrated in all animals injected with either of these 2 vaccines. Vaccines prepared from the 1/6, 1/12 and 1/24 harvest dilutions produced a detectable serological response in 40, 80 and 20 per cent of animals 28 days post-vaccination with corresponding mean HAI titres of 1/19, 1/25 and 1/6. The relationship between vaccine potency and the preinactivation

Fig. 6.2 The 14 and 28 day antibody responses to vaccines prepared from unconcentrated harvest E3

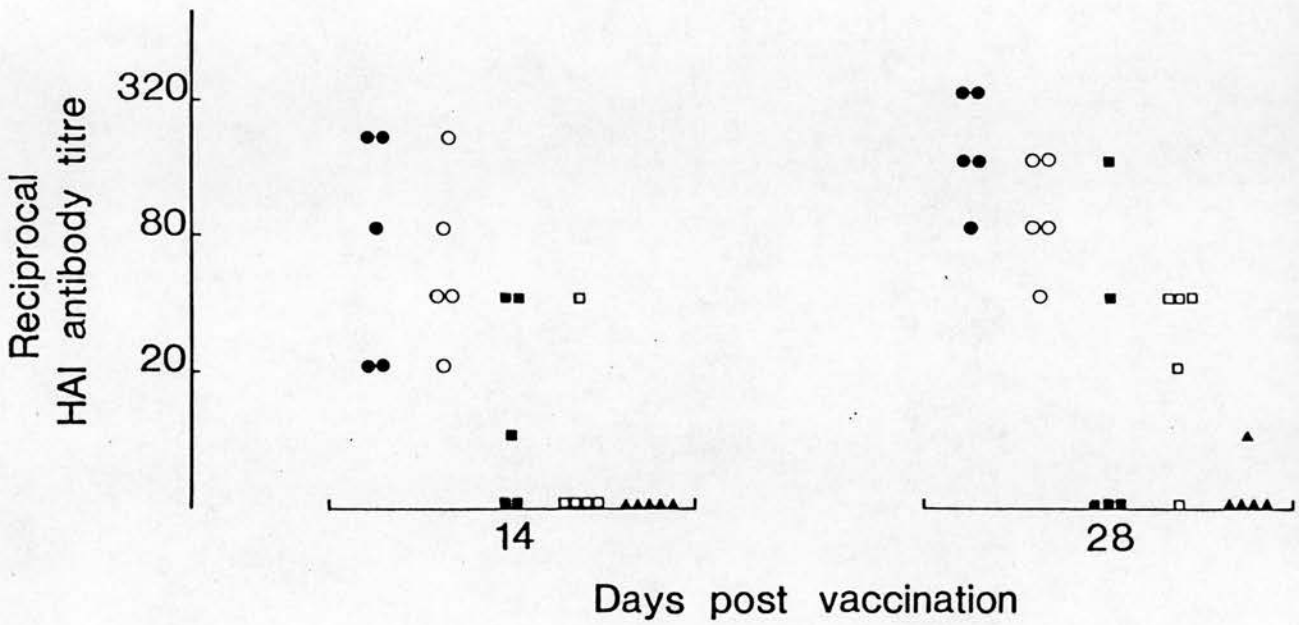
○ 1/3 dilution

■ 1/6 dilution

□ 1/12 dilution

▲ 1/24 dilution

● undiluted


Reciprocal
HAI antibody titre
320
80
20
14
28
Days post vaccination

infectivity titre of the virus harvest is illustrated in Fig. 6.3. The correlation coefficient was +0.769 ($p < 0.001$).

DISCUSSION

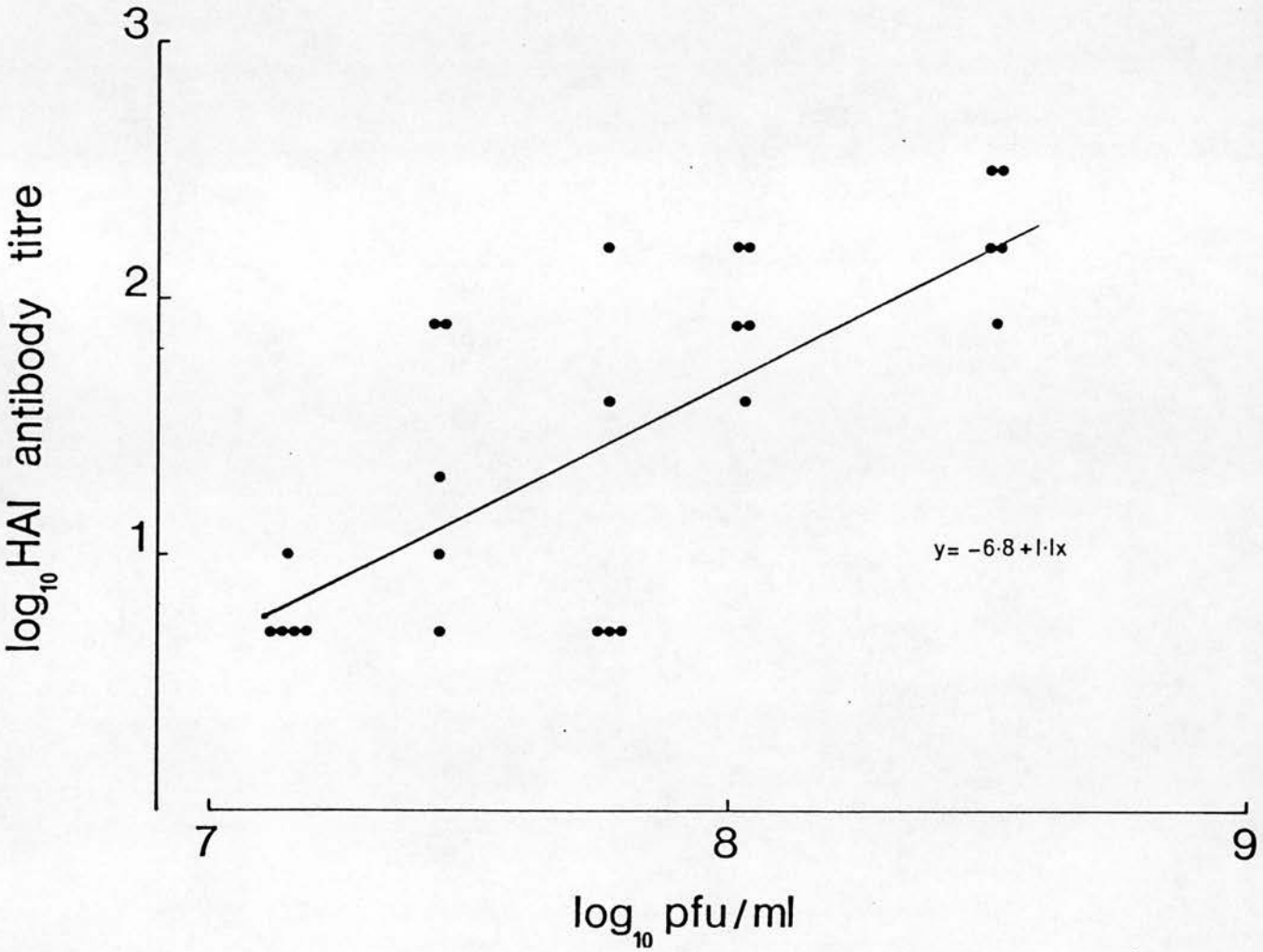
Brotherston and Boyce (1970) reported that methanol treatment of formalinized louping-ill virus propagated in secondary cultures of sheep kidney cells produced experimental vaccines of considerable immunogenicity, and that vaccines prepared from untreated virus were non-immunogenic. To assess the efficacy of methanol precipitation of commercially produced louping-ill virus (propagated in BHK-21 cells) the comparative immunogenicity of vaccines prepared from methanol treated virus harvests and untreated virus harvests was examined.

The concentration of commercially produced virus using methanol was shown to be inefficient because there was no significant difference between the potency of unconcentrated vaccines and the 10- and 5-fold concentrate vaccines. Furthermore, the antibody response to unconcentrated vaccine was significantly higher than the response to the 2.5- and 1.25-fold concentrate vaccines. The percentage of persistently seronegative sheep was also considerably lower in animals vaccinated with the unconcentrated vaccine.

A comparison between the commercial and experimental methods of vaccine production is required in an attempt to understand the apparent success of concentrating virus with methanol in the experimental system, and the overt inefficiency of the same method when applied to an approximately equivalent system.

Louping-ill vaccine is required primarily for the protection of sheep against natural infection and the essential difference between

Fig 6.3 The 28 day HAI titres to vaccines prepared from virus harvests with varying preinactivation infectivity titres


3
2
1
log10HAI antibody titre
y= −6·8 + 1·1x
7
8
9
log10 pfu/ml

the two vaccine production methods is that, experimentally, virus was propagated in sheep tissue (a homologous system) in the absence of serum, whereas commercially virus is propagated in baby-hamster-kidney tissue (a heterologous system) in media containing 10 per cent lamb serum.

The difficulties associated with immunization using virus derived from heterologous tissue suspensions have long been recognized (Traub, 1938; Koprowski and Cox, 1946; Maurer, Walker, Shope, Griffiths and Jenkins, 1946; Edward, 1948b) and the rationale of Brotherston and Boyce (1970) to propagate louping-ill virus in homologous cell culture and to further reduce the amount of foreign protein present by removal of cellular debris may therefore be a particularly important factor contributing significantly to the success of their experimental vaccine.

Commercial propagation of virus using a heterologous cell culture system, media containing serum and the non-removal of cell debris will inevitably incorporate a greater amount of non-viral antigenic material than in harvests derived from homologous culture. Methanol precipitation of commercially produced virus harvests may therefore concentrate not only specific antigen but also non-specific antigens and intermolecular and intramolecular immunogenic competition and interference may result. In addition, concentration of the antigens could be unequal, producing an antigenic imbalance in the final concentrate in favour of non-specific antigen and a corresponding decrease in specific antigenicity of the final preparation.

The use of a heterologous cell system for virus propagation and the contrasting effect of methanol precipitation on the antigenic activity of virus derived from homologous and heterologous cell culture will be discussed further in Chapter Nine.

The potency test specification with respect to commercial louping-ill vaccine states that out of 6 sheep injected with vaccine the sera of 5 must contain HAI antibodies at dilutions of 1/10 or greater. The immunogenicity of vaccines prepared from unconcentrated virus harvests was shown to be a reflection of the preinactivation infectivity titre. Vaccines prepared from virus harvests having an antigenic mass equivalent to titres of 5×10^7 pfu/ml and less produced a serological response in only 80, 40 and 20 per cent of animals. These vaccines would therefore be unacceptable for commercial issue. A serological response was however shown in all animals injected with vaccines prepared from unconcentrated virus harvests having an antigenic mass equivalent to pre-inactivation titres in excess of 10^8 pfu/ml. Such vaccines would therefore be acceptable for commercial issue.

Thus, providing the titre of the source material is in excess of 10^8 pfu/ml, concentration of antigen is an unnecessary step and vaccines of satisfactory potency can be prepared from unconcentrated commercial harvests. Production of such vaccines however demands a constant supply of high titred source material. As the present commercial technique employed for virus propagation is a batch method and titres of individual harvests have been shown to range from 10^8 pfu/ml to 10^6 pfu/ml, to consistently produce vaccines of acceptable potency a method of concentrating viral antigen other than by methanol precipitation would be expedient.

Table 6.1 The 28 day mean reciprocal HAI titres(log_{10}) elicited by concentrated and unconcentrated louping-ill vaccines A-F

Harvest Pool	Mean Infectivity‡ of Harvest Pool	Vaccine Concentration Factor				
		Unconc.	10x	5x	2.5x	1.25x
A	8.2 ± 0.06	1.6	2.6**	1.8	1.5	1.7
B	8.1 ± 0.05	2.0	1.9	1.7	1.5	1.5
C	8.1 ± 0.09	2.3	1.6	1.5	1.2*	1.5
D	8.1 ± 0.07	2.0	1.8	1.7	1.4	1.2
E	8.4 ± 0.02	2.7	2.6	1.8	2.0	1.9
F	8.2 ± 0.04	1.8	1.9	1.8	1.2	1.6

‡ log_{10} pfu/ml

The statistical differences between the titres elicited by the concentrated and the corresponding unconcentrated vaccines are indicated as follows:

* $p < 0.05$ (data analysed by Dunnett's Multiple Comparison test) and

** $p < 0.01$ (data analysed by the Mann-Whitney non-parametric test

Table 6.2 The composite mean reciprocal HAI titres (log_{10}) elicited by concentrated and unconcentrated louping-ill vaccines A-F

Vaccine Concentration Factor	Days post vaccination 7	14	21	28
Unconcentrated	0.92	1.7	2.1	2.1
10x	1.1	1.9	1.9	2.1
5x	0.8	1.6	1.7	1.7
2.5x	0.7	1.5	1.4***	1.5**
1.25x	0.7	1.4	1.5***	1.6*

The statistical differences between the titres elicited by the concentrated and the corresponding unconcentrated vaccines are indicatedas follows:

* $p < 0.05$

** $p < 0.01$ Data analysed by the Dunnett's Multiple Comparison test

*** $p < 0.001$

Table 6.3 The duration of antibody response in sheep vaccinated with vaccines prepared from unconcentrated harvest pools A-D

Vaccine	Reciprocal HAI titres (log_{10}) at days post-vaccination		
	28	70	112
A	1.6	2.2 $\pm$ 0.16	1.5 $\pm$ 0.20
B	2.0 $\pm$ 0.15	2.6 $\pm$ 0.17	2.3 $\pm$ 0.26
C	2.3 $\pm$ 0.17	2.6 $\pm$ 0.10	2.2 $\pm$ 0.16
D	2.0 $\pm$ 0.23	2.1 $\pm$ 0.20	1.5 $\pm$ 0.23

Mean titres $\pm$ s.e.

Table 6.4 The mean reciprocal HAI titres (log_{10}) in sheep injected with vaccines prepared from unconcentrated harvest pool E2 and harvest dilutions

Preinactivation infectivity titre (log_{10} pfu/ml)	Days post-vaccination					
	7	14	21	28	56	84
8.53	0.7	1.8 ± 0.20 **5/5	2.3 ± 0.18 5/5	2.3 ± 0.10 5/5	1.8 ± 0.08 4/4	1.5 ± 0.06 4/4
8.04	0.7	1.7 ± 0.15 5/5	2.3 ± 0.10 5/5	2.0 ± 0.10 5/5	1.5 ± 0.14 4/4	1.4 ± 0.25 4/4
7.74	0.8	1.1 ± 0.20 3/5	1.7 ± 0.18 5/5	1.2 ± 0.30 2/5	1.1 ± 0.22 2/5	0.9 ± 0.17 2/5
7.44	0.7	0.9 ± 0.18 1/5	1.4 ± 0.24 4/5	1.4 ± 0.17 4/5	1.3 ± 0.21 3/4	1.2 ± 0.22 3/4
7.15	0.7	0.7	1.2 ± 0.24 2/5	0.8 ± 0.05 1/5	0,8 ± 0.03 1/5	0,8 ± 0.05 1/5

Mean titres ± s.e.

**Number of sheep responding/number of sheep vaccinated.

CHAPTER SEVEN

CONCENTRATION OF LOUPING-ILL VIRUS BY ULTRAFILTRATION AND POLYETHYLENE GLYCOL PRECIPITATION

INTRODUCTION

For commercial production of louping-ill virus vaccine, virus is propagated in BHK growth media containing 10 per cent LS and the inactivated virus harvest concentrated by methanol precipitation. The present studies have shown however that methanol precipitation does not effectively concentrate commercially produced louping-ill virus harvest. It therefore appeared desirable to investigate concentration of virus antigen by methods other than methanol precipitation.

Commercial propagation of virus using a heterologous cell system with media containing serum and the non-removal of cellular debris will produce virus harvests considerably more diverse in antigenic character than the method of virus harvest preparation described by Brotherston and Boyce (1970). It therefore also seemed appropriate to investigate the concentration of clarified-virus harvests propagated in serum-free media by methanol precipitation and other concentration techniques in an attempt to produce virus antigen concentrates with reduced non-specific antigenic activity.

Several methods of concentrating viruses have been described. The technique of adsorbing virus to salts and molecular gels has been applied to certain viruses, e.g. poliovirus (Sabin, 1932; Schaeffer and Brebner, 1933), fowl plague virus, Newcastle disease

virus and mumps virus (Elford, Chu, Dawson, Dudgeon, Fulton and Smiles, 1948; Frimmhagen and Knight, 1959; Miller and Schelsinger, 1955; Salk, 1941), and enteroviruses (Midulla, Wallis and Melnick, 1965). Concentration by precipitation with polyethylene glycol has been applied to most groups of viruses including the togaviruses (Horzinek, 1969; Della-Porta and Westaway, 1972; Inglot, Chudzio and Albin, 1973; Igarashi, Fukuoka, Sasao, Surimarut and Fukai, 1973). Physical methods of concentration employing ultracentrifugation and ultrafiltration techniques have been described for poliovirus (Guskey and Wolff, 1972), Rift Valley fever virus (Klein, Mahlandt, Bonner and Lincoln, 1971), foot-and-mouth disease virus (Morrow, Whittle and Eales, 1974), murine leukaemia and sarcoma viruses (Rhim, Williams, Huebner and Turner, 1969) and baboon endogenous virus (Weiss, 1980).

The concentration of commercially produced virus harvests and clarified virus harvests propagated in serum-free media by ultrafiltration and precipitation with polyethylene glycol is examined in this chapter. The preparation and comparative physical characteristics of the concentrates are outlined in Part I and the immunogenicity of vaccines prepared from these concentrates and commercially prepared vaccine are described and contrasted in Part II. Particular reference is made to the feasibility of concentrating large volumes of inactivated louping-ill virus, using these methods, for production of vaccine on a commercial level.

PART I PREPARATION AND PHYSICAL CHARACTERISTICS OF THE VIRUS CONCENTRATES

MATERIALS AND METHODS

Virus was propagated in BHK-21 cells cultured in rolling half-gallon bottles in either BHK virus growth media containing 10 per cent LS or in EBA media as described in Chapter Four. Virus was harvested after 72 hours incubation and the serum-free virus harvests were clarified by centrifugation for one hour at 3000 rpm. All live virus harvests were stored at -80°C prior to concentration, and virus inactivation, when required, was performed by treatment with 0.1 per cent formalin at 37°C for 24 hours.

CONCENTRATION OF VIRUS BY PRECIPITATION WITH POLYETHYLENE GLYCOL 6000*

Polyethylene glycol 6000 (PEG) was used throughout. Appropriate amounts of solid PEG were added to live or inactivated virus harvests previously chilled to 4°C. Small volume preparations (40ml) were incubated in an ice bath and shaken vigorously initially and again at regular intervals throughout the incubation. Larger volume preparations (>100ml) were incubated at 4°C and stirred continuously with a magnetic stirrer for the required period. The resulting precipitates were pelleted by centrifugation at 4000g for 60 minutes at 4°C and resuspended in the required volume (usually 1/10 or 1/20 of the starting volume) of either PBS or PBS plus 0.5 per cent (v/v) phenol. The precipitates thus formed were however characteristically sticky and difficult to resuspend. In an attempt to facilitate complete resuspension a live virus concentrate was

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*BDH Chemicals Ltd., Poole.

therefore also subjected to sonication for 30 seconds using a MSE* 150 watt ultrasonic disintegrator.

CONCENTRATION OF VIRUS BY ULTRAFILTRATION

Concentration of live virus by ultrafiltration was not attempted as the method involved the processing of live virus under pressure, and the possibility therefore existed of creating aerosols of live virus.

Concentration of inactivated virus harvests was performed using a stirred cell ultrafiltration system or a hollow fibre ultrafiltration cartridge.

1. Stirred cell ultrafiltration

Small volumes of virus (200ml maximum) were filtered in an Amicon** stirred cell model 202 through a Diaflo membrane type XM300 with a molecular weight cut-off of 300,000. The procedure was operated at $4^{o}C$ with an upstream pressure of 0.68 atm. A serum-free virus harvest was concentrated 50-fold and the commercial virus harvests 10-fold. The time required for completion did not exceed 24 hours.

2. Hollow fibre ultrafiltration cartridge

(a) DOW hollow fibre cartridge: 2000ml of a commercially produced inactivated-virus harvest was concentrated 40-fold using a DOW cartridge having a molecular weight cut-off of 30,000. The virus suspension was drawn through the cartridge using water suction at a pressure of 1.36 atm. High molecular weight material was retained within the cartridge, and solvent and low molecular weight material was directed

*MSE, Crawley.
**Amicon Ltd., High Wycombe.

to waste. The time required to concentrate the virus suspension 40-fold was approximately 2 hours and the procedure was carried out at room temperature. The concentrated virus material was then poured from the cartridge, the cartridge washed twice with PBS plus 0.5 per cent (v/v) phenol and shaken vigorously to release virus adsorbed to the fibres. The washings were added to the virus concentrate. The cartridge was refilled with PBS plus 0.5 per cent (v/v) phenol and rotated overnight at $4^{o}C$. This washing was then also added to the virus concentrate and the volume adjusted to 200ml to give a 10-fold concentrate.

Washing and sterilizing of the cartridge prior to re-use was performed according to the manufacturer's instructions. All the washing solutions were filtered through a 0.45µm filter to remove fibres and thus reduce clogging of the cartridge.

(b) Amicon hollow fibre cartridge: Commercially produced inactivated-virus harvests (maximum volume 9000ml) were concentrated 10-fold using an Amicon Hollow Fibre Concentrator model CH4A. The model was fitted with an Amicon hollow fibre cartridge type HIP100 with a molecular weight cut-off of 100,000. The internal diameter of each fibre within the cartridge was 0.5mm giving a total area for filtration of $0.07m^{2}$. The fibres were constructed of the same non-cellulosic material as the flat Amicon membranes. The cartridge was fitted with an in-line pre-filter to remove cellular debris and thus reduce clogging of the fibre surface. The pre-filter was replaced between each concentration procedure.

In operation the inactivated-virus suspension was drawn from a reservoir through the pre-filter and then through the fibre cartridge

by a built-in peristaltic pump. The pressure within the cartridge was controlled by a back pressure valve at the cartridge outlet. Solvent and low molecular weight species were driven through the fibre walls by the internal cartridge pressure (1-1.75 atm.) and discarded to waste. Concentrated material was returned to the reservoir and recycled until a 10-fold concentrate was approximately achieved. Recirculation of the concentrated material was then arrested and the system cleared of concentrate. The back pressure valve was then completely opened, the filtrate outlet closed and 20ml of PBS recirculated through the system to obtain maximum recovery of concentrate. The volume of the concentrate was then adjusted with PBS to give a 10-fold concentrate. On average one litre of virus harvest was concentrated to 100ml in one hour.

The cartridge was cleaned and resterilized according to the manufacturer's instructions.

CONCENTRATION OF VIRUS BY PRECIPITATION WITH METHANOL

To compare the concentration of virus using PEG precipitation and ultrafiltration with the commercial method of concentration, live and inactivated virus harvests were also concentrated by methanol precipitation as described in the production of commercial vaccine (Appendix 3.1). Concentrated preparations derived from live harvests were however resuspended in PBS without phenol. A live virus concentrate was also subjected to sonication for 30 seconds as described previously.

The unconcentrated live virus harvests, the live virus concentrates (prepared by PEG and methanol precipitation) and the corresponding supernatants were assayed for infectivity and HA

activity as described in Chapter Two. Protein content was determined by the Kjeldahl method.

Inactivated virus concentrates, prepared by concentrating commercially produced virus by methanol precipitation, PEG precipitation and ultrafiltration were examined by immunodiffusion using the double diffusion and the single radial diffusion methods as described in Chapter Two.

RESULTS

CONCENTRATION OF VIRUS BY PRECIPITATION WITH PEG 6000

1. Concentration of virus propagated in BHK virus growth media (10 per cent LS)

The results of concentrating a live commercial virus harvest with PEG 6000 at concentrations ranging from 3-8 per cent (w/v) are given in Table 7.1. The protein content of the unconcentrated virus harvest was 5.8 mg/ml. The precipitates formed were characteristically sticky and some difficulty in resuspending was encountered. With each polymer concentration the virus harvest was concentrated by a factor of 10 with respect to volume.

Precipitation of viral activity as measured by HA activity commenced with a 3 per cent concentration of polymer. Maximum recovery of activity (80 per cent) was achieved following precipitation with PEG concentrations of 6, 7 and 8 per cent. No residual HA activity was detectable in the corresponding supernatants. Maximum recovery of infectivity (24 per cent) was attained with concentrations of 7 and 8 per cent and less than 1 per cent of the original infectivity was detected in the corresponding supernatant.

Precipitation of protein increased with increasing polymer concentrations, ranging from 0.6 to 20 per cent recovery following treatment with 3-8 per cent PEG respectively.

Sonication of a virus concentrate prepared by precipitating virus with a 7 per cent concentration of polymer did not affect HA recovery but did result in an increase in infectivity recovery from 36-58 per cent. No residual HA activity and less than one per cent of the original infectivity were detected in the supernatant.

2. Concentration of virus propagated in serum-free media

The results of concentrating serum-free virus harvests by PEG at concentrations ranging from 3-8 per cent are given in Table 7.2. Prior to concentration the virus harvest was clarified to remove cell debris. The protein content of the unconcentrated harvest was 4.5 mg/ml. The precipitates formed were less viscous than the commercial virus harvest precipitates but again resuspension was difficult. With each polymer concentration the virus harvest was concentrated to a factor of 20 with respect to volume.

Precipitation of HA activity commenced with a 4 per cent concentration of PEG and maximum recovery (80 per cent) was achieved with concentrations of 7 and 8 per cent. Maximum recovery of infectivity (26 per cent) was also similar to that obtained with commercial virus harvest. In contrast to the concentration of commercial virus harvest, however, increasing the polymer concentration did not appreciably increase protein precipitation and the average recovery was 0.6 per cent.

CONCENTRATION OF VIRUS BY ULTRAFILTRATION

Commercially produced louping-ill virus harvest was concentrated 10-fold using either a stirred cell or a hollow fibre ultrafiltration system. Recovery of protein ranged from 70-100 per cent. Inactivated virus propagated in serum-free media was concentrated 50-fold using a stirred cell ultrafiltration system.

CONCENTRATION OF VIRUS BY PRECIPITATION WITH METHANOL

The results of concentrating live virus according to the commercial method of concentration (described in Appendix 3.1) are shown in Table 7.3. Maximum recovery of virus activity as determined by HA and infectivity was 20 and 0.4 per cent respectively. No residual HA activity and less than 0.001 per cent of the original infectivity were detected in the supernatant. Sonication of a concentrated virus preparation did not enhance recovery of viral activity. The recovery of protein following methanol precipitation of 3 inactivated virus harvests ranged from 7-14 per cent. Precipitation of live virus propagated in EBA serum-free media was not investigated.

Inactivated virus concentrates prepared by methanol precipitation, PEG precipitation and ultrafiltration were examined by immunodiffusion using the methods described in Chapter Two. A 7 per cent concentration of PEG was employed and ultrafiltration was carried out using a hollow fibre system. Sonicated preparations of the methanol and PEG concentrates were also tested.

No reaction was detected between a 1/8 dilution of sheep hyperimmune serum and the methanol and PEG inactivated virus concentrates

using a double diffusion method (Fig. 7.1a). Inactivated virus concentrated 10-fold and 5-fold by ultrafiltration however showed a reaction of identity with the reference antigen producing a single continuous line of precipitin (Fig. 7.1b).

A normal mouse brain preparation, the concentrated BHK cellular extracts (concentrated 10-fold by each of the concentration methods) and the BHK virus growth media did not react with the antibody.

The single radial diffusion method was shown to be of limited value in attempting to quantify the relative antigenic activity of each of the three concentrates.

Optimal conditions were described when antibody was incorporated into the gel at a final dilution of 1/100 and a 1/5 dilution of reference antigen used. The concentrated BHK cellular extracts and normal mouse brain preparation did not react with antibody. Only virus harvest concentrated 10-fold by ultrafiltration produced a ring of precipitin (Fig. 7.2), thus as the PEG and methanol 10-fold virus harvest concentrates did not react with antibody, the comparative antigenicity of the three concentrates could not be determined.

DISCUSSION

Concentration of commercially produced live louping-ill virus by methanol precipitation resulted in almost total loss of viral activity with regard to both haemagglutination and infectivity. A likely explanation is that methanol caused physical alteration to, or denaturation of, the virus particle. It is also possible therefore that the activity of surface antigenic sites was similarly

Fig. 7.1 Immunodiffusion of concentrated virus preparations

(a) Ab anti louping-ill sheep serum

Mb infected mouse brain

M methanol 10-fold virus antigen concentrate

P PEG 10-fold virus antigen concentrate

BHKM methanol concentrated BHK cell extract

BHKP PEG concentrated BHK cell extract

(b) A10x ultrafiltration 10-fold virus antigen concentrate

A5x ultrafiltration 5-fold virus antigen concentrate

BHKA ultrafiltration concentrated BHK cell extract

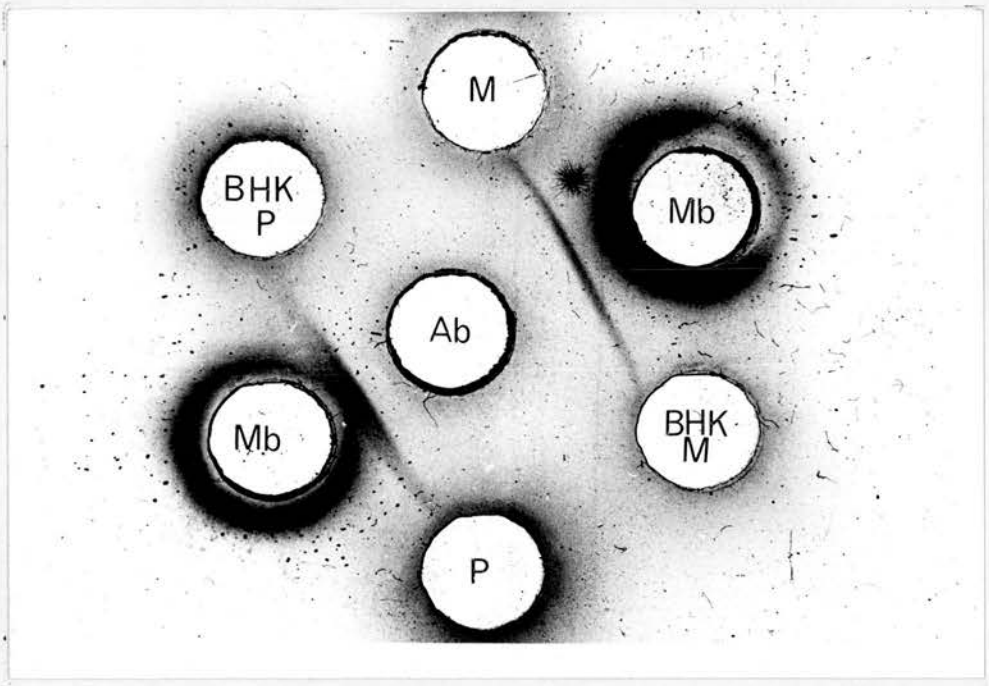


Fig. 7.1a

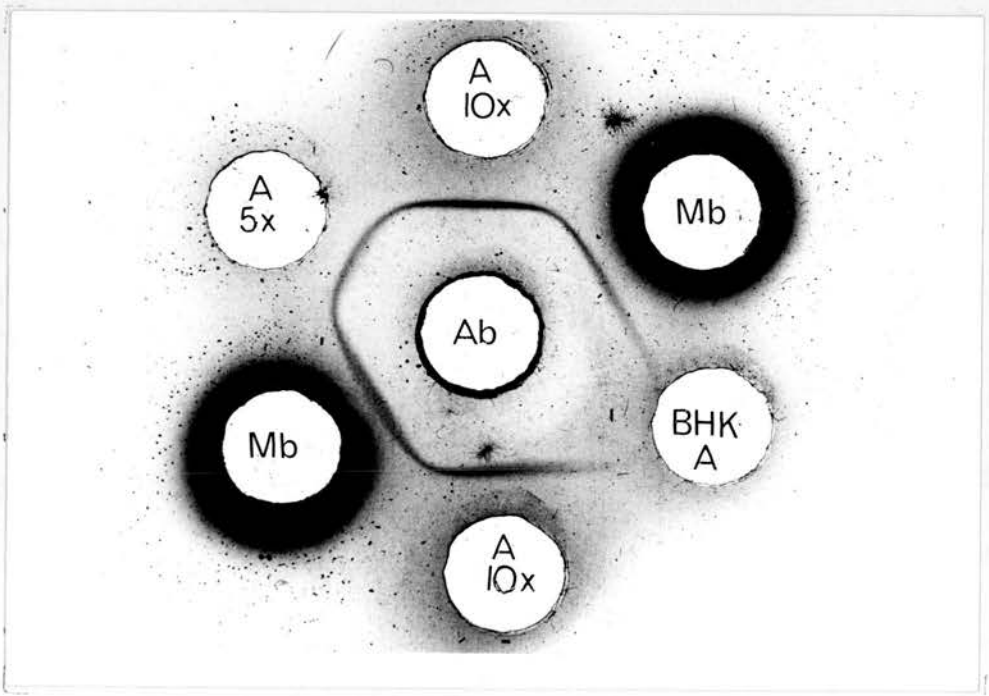


Fig. 7.1b

Fig. 7.2 Radial immunodiffusion of concentrated virus preparations

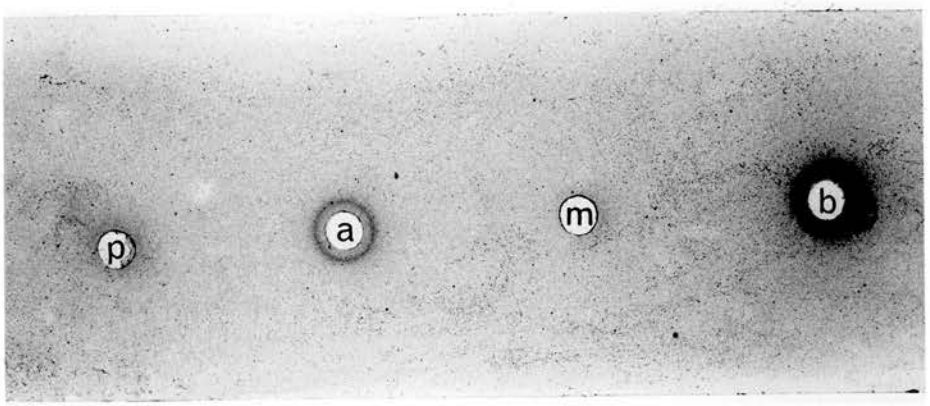
p PEG 10-fold virus antigen concentrate

a ultrafiltration 10-fold virus antigen concentrate

m methanol 10-fold virus antigen concentrate

b infected mouse brain

Antibody incorporated into the gel at a final dilution of 1/100


p
a
m
b

diminished following treatment with methanol. The need for an alternative method of concentrating virus is thus further emphasised.

Of the wide variety of concentration methods available precipitation with polyethylene glycol 6000 and ultrafiltration were chosen for further examination.

Polyethylene glycol is a high molecular weight, water soluble polymer and a method of concentrating and purifying viruses using an aqueous PEG dextran sulphate 2-phase system was first developed by Philipson, Albertsson and Frick (1960), and Norrby and Albertsson (1960). Studies by Hebert (1963) and Leberman (1966) indicated that PEG alone or in combination with sodium chloride could be effectively used to concentrate plant viruses and bacteriophages. Subsequently this method of concentration has been applied to representatives of the Rhabdoviridae (McSharry and Benzinger, 1970); the Herpetoviridae (Adams, 1973); the Retroviridae (Bronson, Elliott and Ritzi, 1975), the Myxoviridae (Kanarek and Tribe, 1967), the Picornaviridae (Wagner, Card and Cowan, 1970), and the Papovaviridae (Friedmann and Haas, 1970). Precipitation of the Togaviridae with PEG has been described by Horzinek (1969); Della-Porta and Westaway (1972); Igarashi, Fukuoka, Sasao, Surimarut and Fukai (1973); Inglot, Chudzio and Albin (1973), and Heinz and Kunz (1977a).

Heinz and Kunz (1977a) studying the concentration and purification of tick-borne encephalitis virus demonstrated that precipitation with PEG at concentrations of 5-8 per cent resulted in maximum recoveries of HA activity and infectivity (100 and 60 per cent respectively), and Horzinek (1969) reported that concentration of Venezuelan equine encephalitis virus using a polymer concentration

of 10 per cent combined with a sodium chloride concentration of 0.25M resulted in 100 per cent recovery of infectivity. Infectivity of 3 other togaviruses ranged from 23-60 per cent. An average recovery of 60 per cent of infectivity and 100 per cent HA activity was obtained following precipitation with a PEG concentration of 8 per cent with Japanese B encephalitis, Kunjin, Murray Valley encephalitis, St. Louis encephalitis and West Nile viruses (Della-Porta and Westaway, 1972) and Igarashi, Fukuoka, Sasao, Surimarut and Fukai (1973) demonstrated complete recovery of HA activity and a 30-75 per cent recovery of the infectivity of Japanese encephalitis virus following treatment with 8 per cent PEG and a sodium chloride concentration of 0.5M.

Precipitation of louping-ill virus was examined using concentrations of PEG ranging from 3-8 per cent. Concentration by precipitation with polymer at concentrations of 7 and 8 per cent resulted in maximum recovery of HA activity (80 per cent) and infectivity (24-36 per cent) although the precipitates formed were viscous and resuspension was difficult. Sonication of a virus concentrate precipitated with a 7 per cent concentration of polymer increased infectivity but had no effect on HA recovery.

Wagner, Card and Cowan (1970) demonstrated that optimal recovery from foot-and-mouth disease virus was achieved with virus propagated in serum-free media. Addition of serum to the growth medium decreased infectivity recovery and produced precipitates that were difficult to dissolve. It was concluded that such a loss in infectivity was probably due to entrapment of the virus in proteinaceous debris.

PEG precipitation of Rous sarcoma virus propagated in media containing 10 per cent serum and 10 per cent TPB also caused aggregation of particles (Bronson, Elliott and Ritzi, 1975), and it was proposed that proteinaceous materials from medium supplements were responsible for aggregation and the subsequent loss in foci-forming units.

As sonication of a louping-ill virus concentrate increased infectivity recovery it is possible that loss of detectable infectivity recovery following precipitation of virus with PEG may also be attributed to aggregation of virus particles caused by medium supplements.

The technique of ultrafiltration has been employed for a number of years to concentrate a variety of substances within the pharmaceutical and food industries. More recently Van Wezel (1974) described its use for concentrating tetanus toxin, poliovirus and vaccinia virus on a large scale for vaccine production.

Concentration of large volumes of foot-and-mouth disease virus using an Amicon hollow-fibre ultrafilter resulted in CF antigen recoveries of between 70 and 100 per cent (Morrow, Whittle and Eales, 1974) and Klein, Mahlandt, Bonner and Lincoln (1971), using a stirred-cell ultrafiltration system to concentrate Rift Valley fever virus, obtained recoveries ranging from 40-100 per cent. Ultra-filtration of murine leukemia and sarcoma viruses produced quantitative concentration of antigen activity as measured by CF activity and focus forming activity (Rhim, Williams, Huebner and Turner, 1969).

Concentration of live louping-ill virus using ultrafiltration techniques was not attempted due to the hazards involved. Concentration of inactivated virus harvest was however easily accomplished and no difficulties were encountered.

Immunodiffusion tests showed that only virus concentrated by ultrafiltration reacted with antibody and a single continuous line of identity with the reference antigen was produced. No reaction was observed between antibody and virus harvest concentrated by methanol and PEG precipitations. A valid comparison of the antigenic activity of the 3 concentrates using this test cannot be established as it is possible that precipitation of virus with methanol and PEG (particularly the latter) resulted in aggregation of virus and the production of non-diffusable antigens.

The 2 methods of concentration under consideration both appear to be readily adaptable to the concentration of large volumes of virus. A possible advantage of the PEG method was that concentration also resulted in partial purification. A disadvantage of this technique however was that the precipitates formed were difficult to dissolve and aggregation of virus particles occurred; this may have an adverse effect on the immunogenicity of subsequent vaccine.

Ultrafiltration is a physical method of concentration and the probability of denaturing the virus particle is small. A hollow fibre ultrafiltration system is particularly well suited for scale-up for the processing of large volumes of virus. The cartridges can be re-used repeatedly without loss of filtration efficiency and the concentration procedure is simple and inexpensive in terms of both operation and manpower.

The comparative immunogenicity of vaccines prepared from PEG concentrates, ultrafiltration concentrates and methanol concentrates will be examined and discussed in Part II.

Table 7.1 Precipitation of louping-ill virus harvest* with varying concentrations of polyethylene glycol 6000

PEG conc. (%)	Sample	Volume (ml)	Infectivity $\log_{10}$ pfu/ml	% recovery	Reciprocal HA titre	% recovery	Protein mg/ml	% recovery
	Original Material	40	8.17		256		5.8	
3	Concentrate	4	2.84	-	256	10	0.38	0.6
	Supernatant	40	7.11		128		5.84	
4	Concentrate	4	7.80	4	1024	40	1.05	1.8
	Supernatant	40	5.63		32		5.20	
5	Concentrate	4	8.32	14	1024	40	1.46	2.5
	Supernatant	40	4.43		<4		5.42	
6	Concentrate	4	8.32	14	2048	80	5.50	9.4
	Supernatant	40	4.17		<4		4.12	
7	Concentrate	4	8.55	24	2048	80	3.76	6.4
	Supernatant	40	4.32		<4		4.23	
8	Concentrate	4	8.56	24	2048	80	11.6	20.0
	Supernatant	40	ND		<4		3.69	

*Virus propagated in BHK virus growth media

ND = not tested

Table 7.2 Precipitation of louping-ill virus harvest* with varying concentrations of polyethylene glycol 6000

PEG conc. (%)	Sample	Volume (ml)	Infectivity $\log_{10}$ pfu/ml	% recovery	Reciprocal HA titre	% recovery	Protein mg/ml	% recovery
	Original Material	40	7.68		512		4.5	
3	Concentrate	2	-	-	4	-	0.43	0.47
	Supernatant	40	7.47		256		3.70	
4	Concentrate	2	6.87	0.7	1024	10	0.70	0.77
	Supernatant	40	6.85		128		3.60	
5	Concentrate	2	7.38	2.5	1024	10	0.38	0.42
	Supernatant	40	6.11		64		3.60	
6	Concentrate	2	7.83	7.0	2048	20	0.54	0.60
	Supernatant	40	6.07		16		3.45	
7	Concentrate	2	8.38	26.0	8096	80	0.69	0.76
	Supernatant	40	5.65		<4		3.78	
8	Concentrate	2	8.40	26.0	8096	80	0.67	0.74
	Supernatant	40	5.51		<4		3.33	

*virus propagated in serum-free media

Table 7.3 Concentration of louping-ill virus harvest* by methanol precipitation

Sample	Volume (ml)	Infectivity $\log_{10}$ pfu/ml	% recovery	Reciprocal HA titre	% recovery
Original material	100	8.69		256	
Concentrate	10	6.30	0.400	512	20
Concentrate (sonicated)		6.27	0.400	512	20
Supernatant	100	3.46	0.001	4	-

*Virus propagated in BHK virus growth media

PART II THE IMMUNOGENICITY OF VACCINES CONCENTRATED BY POLYETHYLENE GLYCOL PRECIPITATION, ULTRAFILTRATION AND METHANOL PRECIPITATION

MATERIALS AND METHODS

Inactivated virus propagated in either BHK virus growth media containing 10 per cent LS or EBA serum-free media was concentrated by each of the 3 methods described in Part I.

The concentrates were stored at -20°C. When required vaccines were prepared from each of the concentrates and concentrate dilutions by emulsification with Bayol F and Falba oil adjuvant in a ratio one part concentrate to two parts adjuvant to give, unless otherwise stated, a final volume of 15ml. Emulsification was carried out using a MSE bench type overhead homogeniser.

The pre-blend potency vaccines and final potency vaccine derived from the commercial vaccine production 1978-79 (Appendix 3.1) were also made available for comparative purposes.

Vaccine potency was determined initially in either mice or chickens and on the basis of the results obtained, selected vaccines were then further assayed in sheep and cattle.

1. ANTIBODY RESPONSE IN MICE

Schneider Swiss White random bred mice, C57 black inbred mice, athymic Nu. Nu. mice and litter mates were used to assess vaccine potency. The mice were bred and housed at the Moredun Research Institute and were inoculated at approximately 3 weeks of age with 0.5ml of vaccine. The vaccine was injected intra-peritoneally (i.p.). Athymic mice and litter mates were housed in plastic isolators. Animals vaccinated with pre-blend potency vaccines derived from the

1978-79 commercial vaccine production were revaccinated at 21 days with a further 0.5ml, and 3 weeks later were killed and the blood collected by heart puncture. Animals vaccinated with the experimental vaccines received one dose of vaccine only, were killed at 21 days and the blood collected. The blood was allowed to clot, the serum collected and tested for HAI antibody to louping-ill as described in Chapter Two.

2. ANTIBODY RESPONSE IN CHICKENS

One-day-old chicks were purchased from a local hatchery and housed at the Moredun Research Institute. At 10 days of age they were injected intra-muscularly (i.m.) with 0.5ml of vaccine. The birds received either the pre-blend potency vaccines (commercial vaccine production 1978-79) or experimental vaccine prepared from virus propagated in serum-free media and concentrated by stirred cell ultrafiltration. Each vaccine was injected into 5 chicks. After 14 days the birds were bled from the right jugular vein and a further 0.5ml of vaccine injected i.m. They were then bled weekly for 3 weeks, 2ml of blood being withdrawn from each bird on each occasion. The blood was allowed to clot, the serum removed and tested for HAI antibody activity (Chapter Two).

3. ANTIBODY RESPONSE IN SHEEP

Various breeds of sheep, aged between 7 months and 2 years, and born either at the Moredun Research Institute or in other areas free of ticks, were used to assess vaccine potency. Prior to vaccination all the sheep were tested for the presence of louping-ill HAI serum antibodies and all were found to be seronegative. The animals were injected sub-cutaneously (s.c.) behind the right

shoulder with 1ml of vaccine and each vaccine was injected into groups of 4, 5, 6 or 12 sheep depending on availability. Animals vaccinated with batches of final potency commercial vaccine were bled weekly for 3 weeks, revaccinated with a further 1ml dose and then bled weekly for a further 4 weeks. Sheep vaccinated with the experimental vaccines received one 1ml dose only and were bled weekly for 4 weeks following inoculation. On each occasion approximately 10ml of blood was withdrawn from each animal.

The blood was allowed to clot and the serum collected and tested for HAI antibody activity.

4. ANTIBODY RESPONSE IN CATTLE

Thirteen Jersey calves aged between 12 and 18 months and 21 Hereford cows were inoculated with vaccine. Prior to vaccination the animals were tested for the presence of louping-ill HAI serum antibodies and all were found to be seronegative. The animals were injected s.c. in the right side of the neck and each vaccine was injected into groups of 4, 5 or 6 animals depending on availability. Calves vaccinated with a batch of final potency commercial vaccine received an initial 2ml dose, followed by a further 2ml after 21 days. Animals inoculated with the experimental vaccines received an initial 1ml dose followed 28 days later by a second 1ml dose. Following revaccination the animals were bled weekly for at least 4 weeks, approximately 10ml of blood being withdrawn from each animal. The blood was allowed to clot, the sera removed and tested for HAI activity.

Five of the calves inoculated with a vaccine prepared from virus propagated in BHK virus growth media containing 10 per cent LS

and concentrated by ultrafiltration were subjected to hypersensitivity tests 176 days after initial vaccination. The vaccine used for the tests had been prepared on a commercial scale and had been concentrated 10-fold using an Amicon Hollow Fibre Ultrafilter. Calves X846 and X849 were injected s.c. in the left side of the neck with 2ml and 1ml respectively of the emulsified vaccine. Calves X843, X845 and V516 were injected s.c. with 5ml, 2.5ml and 1ml respectively of the 10-fold concentrate (not emulsified) and intradermally (i.d.) with 0.5ml. The calves were observed for a 4-hour period immediately following injection and skin thickness measured at 0.5, 2.5, 24, 48 and 96 hours.

Statistical analysis

Reciprocal HAI titres were transformed into logarithms to the base 10. Titres of <10 were deemed to have a logarithmic value of 0.7. The data were analysed using either a Mann-Whitney non-parametric test or a 2 sample t-test.

RESULTS

1. ANTIBODY RESPONSE IN MICE

Commercial vaccine

The 1978-79 pre-blend potency vaccines concentrated 10- and 2.5-fold were injected into both the random bred and inbred strains of mice. The vaccines were prepared and tested immediately following production of the 10-fold concentrates. The mice were inoculated with 2 0.5ml doses of vaccine and each vaccine was injected into 10 mice. The mean reciprocal 42-day HAI titres are given in Table 7.4.

There was no significant difference between the serological responses detected in the random-bred and the inbred strains of mice. The maximum mean antibody titres following vaccination with the 10- and 2.5-fold concentrate vaccines was 1/25 and 1/8 respectively.

Experimental vaccines

Experimental vaccines were routinely assessed using the random-bred strain of mice only. A 50-fold concentrate (BAU) prepared from virus propagated in serum-free media and concentrated by stirred cell ultrafiltration was diluted accordingly with PBS plus 0.5 per cent (v/v) phenol to give 10-, 5-, 2- and 1-fold concentrates. Vaccines were prepared and tested immediately following production of the 50-fold concentrate. Each vaccine was injected into 10 mice. Following a single dose of 50-, 10-, 5-, 2- and 1-fold concentrate vaccines the mean 21-day HAI titres were 1/630, 1/316, 1/256, 1/8 and 1/6 respectively (Table 7.5). The preinactivation infectivity titre of the unconcentrated harvest was 1.2×10^8 pfu/ml and the HA titre was 1/512.

The serological response following a single dose of the 10-fold BAU concentrate vaccine was therefore approximately 13-fold higher than the response following a double vaccination of commercial vaccine of equivalent concentration. Preparation of the BAU concentrate vaccine differed from that of the commercial vaccine in 2 respects; the method of virus propagation and the concentration technique. The following experiments were therefore performed in an attempt to determine whether the rationale of growing virus in serum-free media, thereby reducing non-specific antigenic competition and interference, and/or the method of virus antigen concentration

were contributory to the immunogenicity of the vaccine.

(1) Non-specific antigenic interference

(a) Addition of media supplements

Lamb serum and TPB were treated with 0.1 per cent formalin at 37°C for 24 hours. A mixture containing equal volumes of serum and TPB was likewise formalinized. The 50-fold virus antigen concentrate (BAU) was diluted to a 10-fold concentrate with each of these formalinized preparations. The concentrate was also diluted with PBS plus 0.5 per cent (v/v) phenol. Vaccines were prepared and each injected into 10 Swiss White mice. The addition of formalinized supplements did not affect significantly the immunogenicity of the BAU concentrate vaccine (Table. 7.6).

(b) Addition of methanol concentrated BHK cellular extract

BHK-21 cells were grown in rolling culture in BHK virus growth media containing 10 per cent LS. After 3 days incubation at 37°C the cell culture was frozen and thawed 3 times and then treated with a 1/1000 concentration of formalin. The formalinized cell extract was concentrated 10-fold by methanol precipitation. The 50-fold BAU concentrate was diluted to a 10-fold concentrate with either the 10-fold methanol concentrated BHK cell extract or PBS plus 0.5 per cent (v/v) phenol. Vaccines were prepared accordingly and each injected into 10 mice. The addition of methanol concentrated BHK cellular extract did not affect significantly the immunogenicity of the BAU concentrate vaccine (Table 7.6).

(2) Concentration of virus propagated in serum-free media by methanol precipitation and PEG precipitation

The serum-free virus harvest (preinactivation infectivity titre of 1.2×10^8 pfu/ml and HA of 1/512) was also concentrated 10-fold by PEG precipitation and methanol precipitation. The resulting 10-fold concentrates (BAP; BAM) were diluted with PBS plus 0.5 per cent (v/v) phenol to give 5- and 1-fold concentrates; the 50-fold BAU concentrate was similarly diluted. Vaccines were prepared and each injected into 5 or 7 mice. The 21-day HAI results are shown in Table 7.7. Statistical analysis of the results using the Mann-Whitney non-parametric test indicated that the antibody responses evoked by 10- and 5-fold BAU concentrate vaccines were significantly higher ($p < 0.01$; $p < 0.05$) than the response produced by the 10- and 5-fold BAP and BAM concentrate vaccines. The maximum mean HAI antibody titre was 1/501 and was elicited by 10-fold BAU concentrate vaccine. The lowest mean HAI titres were consistently detected in response to the methanol concentrated vaccines.

The results indicated therefore that immunogenicity was significantly affected by the technique of concentration employed and was not influenced by the presence of cellular debris and proteinaceous material.

The immunogenicity of vaccines prepared by propagating virus in BHK growth media containing 10 per cent LS and concentrated by ultrafiltration was therefore investigated and a comparative assessment with respect to the PEG and methanol methods of concentration determined.

(3) Concentration of virus propagated in BHK virus growth media containing 10 per cent LS

A virus harvest (preinactivation infectivity titre 4 x 10^8 pfu/ml) propagated in BHK virus growth media containing 10 per cent LS was concentrated 10-fold by PEG precipitation (LSP), methanol precipitation (LSM), and ultrafiltration (LSU). The concentrates were diluted with PBS plus 0.5 per cent (v/v) phenol, vaccines prepared and each inoculated into 5 or 3 mice. The 21-day HAI titres are given in Table 7.8. The results were analysed using a Mann-Whitney non-parametric test; the serological responses evoked by the 10- and 5-fold LSU concentrate vaccines were significantly higher ($p < 0.05$) than the responses produced by the PEG and methanol vaccines of equivalent concentration. The maximum mean antibody titre was 1/398 and was produced in response to 10-fold LSU concentrate vaccine.

There was no significant difference between the serological responses produced by vaccines concentrated by ultrafiltration and prepared from virus propagated in either serum-free media or in BHK virus growth media (Tables 7.7, 7.8).

The antibody response in athymic Nu Nu mice and litter mates

Vaccination of athymic Nu Nu mice with either 2 doses of commercial vaccine or a single dose of experimental vaccine did not produce a detectable antibody response. Vaccination of the hairy litter mates with 2 doses of commercial vaccine or a single dose of BAU concentrate vaccine produced a mean antibody response of 1/10 or 1/794 respectively. The results suggest that louping-ill antigen is a thymus-dependent antigen.

2. ANTIBODY RESPONSE IN CHICKENS

Commercial vaccine

To assess the antibody response in chickens to commercial louping-ill vaccine the pre-blend potency vaccines (1978-79) were injected i.m. into 10-day-old birds. The chicks were inoculated with 2 doses of vaccine, the total volume being 1ml. Vaccines were tested immediately following emulsification of the virus antigen concentrate.

The maximum mean HAI titres 14 days following primary vaccination and 21 days following revaccination were 1/13 and 1/40 respectively (Table 7.9).

Experimental vaccine

A 50-fold virus antigen concentrate (BAU) was diluted and vaccines prepared. Each vaccine was inoculated into 5 chicks as described previously. The results are given in Table 7.9. The mean HAI titres evoked by the 10-fold concentrate vaccine 14 days after vaccination and 21 days following revaccination were 1/1995 and 1/19952 respectively.

3. ANTIBODY RESPONSE IN SHEEP

Commercial vaccine

The antibody responses in sheep to the 6 final batches of commercial vaccine (concentration factor between 2- and 5-fold) are illustrated in Table 7.10. The vaccines were tested immediately following emulsification of the virus antigen concentrates. Prior to emulsification the concentrates had been stored at -20^{o}C for periods ranging from 6-22 months. The mean antibody titres

following a single inoculation and a double inoculation ranged from 1/6 to 1/50 and 1/8 to 1/200 respectively.

Experimental vaccine

A 50-fold virus antigen concentrate (BAU) which had been stored for 4 weeks at -20°C was diluted and vaccines prepared. Each vaccine was injected into 4 7-month-old Greyface sheep. The sheep received one inoculation of 1ml only. The 28-day HAI results are shown in Table 7.11. All animals injected with the 10- and 5-fold BAU concentrate vaccines were seropositive at 14 days and the mean 28-day antibody titres were 1/251 and 1/129 respectively. Titres remained relatively constant for up to 126 days post-vaccination (Table 7.11).

Vaccination of mice with the experimental louping-ill vaccines indicated that immunogenicity was dependent on the technique of concentration and independent of the method of virus propagation. To test the validity of this observation in sheep a virus harvest propagated in BHK virus growth media containing 10 per cent LS and with a pre-inactivation infectivity titre of 10^8 pfu/ml and HA activity of 1/128 was concentrated by methanol precipitation (LSM), PEG precipitation (LSP), and by hollow fibre ultrafiltration (LSU). The 10-fold virus concentrates were diluted as described previously, vaccines prepared and each injected into 5 9-month-old Crossbred Suffolk and Blackface lambs. The 28-day HAI results are shown in Table 7.12. All animals inoculated with 10- and 5-fold LSU concentrate vaccine were seropositive by 14 days and the 28-day mean HAI titres were 1/126 and 1/251 respectively. Twenty-eight days

after vaccination with the PEG and methanol 10-fold concentrate vaccines, 75 and 80 per cent of animals respectively were seropositive with corresponding mean antibody titres of 1/63 and 1/50.

It is apparent therefore that concentration of virus by ultrafiltration produces vaccines of higher immunogenicity than the commercial method of concentration or by precipitation with PEG.

To assess the efficiency of scaling up the ultrafiltration process for commercial production, a total 18 litres of virus suspension propagated in BHK virus growth media containing 10 per cent LS was concentrated using an Amicon CH4 hollow fibre filter. The maximum volume of virus concentrated in one run was 9 litres. The harvest was concentrated to 1800ml and then diluted with PBS plus 0.5 per cent (v/v) phenol to give a 4.5-fold concentrate. This was divided into 2 lots of equal volume and each emulsified with adjuvant using an Atomix* laboratory blender to give 2 x 6000ml batches of final vaccine. The final vaccine was distributed in 50ml volumes in bottles fitted with rubber flanges and aluminium seals.

One bottle was withdrawn at random from each batch and injected into 6 12-month-old Blackface ewes. The sheep received one injection of 1ml. All animals were found to be seropositive 14 days after inoculation and the mean 28-day HAI titres detected in sheep vaccinated with Batch A and Batch B vaccine were 1/316 and 1/158 respectively. Titres remained relatively constant for up to 126 days post-vaccination (Table 7.13).

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*MSE, Crawley.

4. ANTIBODY RESPONSE IN CATTLE

Commercial vaccine

Four calves were inoculated with 2 2ml doses of commercial vaccine Batch I. At 21 days post-primary vaccination antibody was detected in only 2 animals and the mean HAI titre was 1/16. Twenty-eight days following revaccination 3 of the 4 animals were seropositive and the mean HAI titre was 1/32, but 67 days later titres were reduced to undetectable levels in 3 animals and the mean HAI titre was 1/8.

Experimental vaccine

A virus harvest propagated in BHK virus growth media containing 10 per cent LS and with a pre-inactivation infectivity titre of 10^8 pfu/ml and an HA titre of 1/128 was concentrated 10-fold by methanol precipitation (LSM), and hollow fibre ultrafiltration (LSU). Vaccines were prepared and each injected into 5 or 6 heifers. Two 1ml doses of vaccine were administered as indicated in Table 7.14. At 28 days post-primary vaccination all animals inoculated with the LSM concentrate vaccines were seronegative; 33 and 20 per cent of animals injected with the 5- and 2.5-fold LSU concentrate vaccines were seropositive with mean HAI titres of 1/10 and 1/13 respectively. Twenty-eight days following revaccination all animals inoculated with the LSU concentrate vaccines were seropositive and the mean HAI titres were 1/501 and 1/158 respectively. Antibody was detected in only 20 and 40 per cent of animals inoculated with 2 doses of the LSM concentrate vaccines and the mean 56-day HAI antibody titres were 1/6.

Vaccines concentrated 10-and 5-fold by ultrafiltration (LSU) were also injected into 9 12- to 18-month-old calves. At 28 days post-vaccination an antibody response was detected in all animals receiving the 10-fold concentrate vaccine and in 50 per cent of those receiving the 5-fold concentrate vaccine with corresponding mean antibody titres of 1/25 and 1/10. Four weeks after re-vaccination all animals were seropositive and mean HAI titres were 1/398 and 1/158 (Table 7.15).

Although no untoward reactions were observed following inoculation of cattle with vaccine concentrated by ultrafiltration production of the vaccine does involve propagation of virus in media containing 10 per cent lamb serum and concentration of virus by ultrafiltration also concentrates the serum. It therefore appeared pertinent to assess if the cattle had been hypersensitized. Five of the 9 calves were therefore given a further injection of a vaccine concentrated 10-fold by hollow fibre ultrafiltration. Calves X846 and X849 were re-injected s.c. with 2ml and 1ml respectively of the emulsified vaccine, receiving therefore 0.67ml and 0.33ml respectively of the 10-fold concentrate. Calves X843, X845 and Y516 were injected s.c. with 5ml, 2ml and 1ml respectively of the 10-fold concentrate. In addition all the animals were injected i.d. with 0.5ml of the 10-fold concentrate.

Thirty minutes following injection calf X845 showed signs of respiratory distress manifested by rapid and shallow breathing and serious lacrimation and swelling around the eyes. A plaque 30mm in diameter was evident at the site of inoculation, within 3 hours however the animal appeared to have recovered completely. Immediately

following injection skin thickness readings increased in all animals except calf X846. Maximum readings were detected 30 minutes following injection and the fold increase ranged from 9 (calf X843) to 3 (calf X849). Skin thickness gradually decreased over the following 96 hours.

DISCUSSION

The immunogenicity of vaccines prepared from virus grown in either serum-free media or media containing 10 per cent serum and concentrated by methanol precipitation, PEG precipitation or ultrafiltration was examined and compared with the commercial 1978-79 louping-ill vaccine.

The serological responses in mice, sheep and cattle to a single dose of vaccine prepared from virus propagated in both types of media and concentrated 10- and 5-fold by ultrafiltration were consistently higher than the responses evoked by a single dose of experimental vaccine concentrated 10- and 5-fold with PEG or methanol or a double dose of commercial louping-ill vaccine. Furthermore, in contrast to vaccination with a single dose of the PEG and methanol experimental vaccine (10- and 5-fold concentrate), all sheep inoculated with a single dose of vaccine similarly concentrated by ultrafiltration, responded to vaccination within 14 days. Some sheep (17 - 83 per cent) injected with the 1978-79 final batches of commercial vaccine were found to be persistently seronegative even after administration of a second dose.

The responses evoked by vaccines prepared by ultrafiltration of virus harvests propagated in either of the growth media described were not significantly different and the immunogenicity of the serum-free vaccine was not affected by the addition of media

supplements or cellular extracts. Concentration of virus propagated in both types of media by methods other than ultrafiltration did however decrease immunogenicity. It would therefore appear that these concentration methods are less efficient with regard to recovery of viral antigen than the ultrafiltration technique. Vaccine immunogenicity thus appears to be dependent on the method of virus antigen concentration and is unaffected by the presence of non-specific antigenic determinants in the virus antigen concentrate.

It was apparent from the studies described in Part I that concentration of live virus by PEG precipitation resulted in a high recovery of HA activity but with a corresponding loss in infectivity. Sonication of the virus concentrate increased infectivity recovery, and it was proposed that the loss in detectable infectivity was attributable, at least in part, to aggregation of virus particles.

PEG precipitation of louping-ill virus propagated in both types of media produced vaccines of lower immunogenicity than the corresponding vaccines concentrated by ultrafiltration. If aggregation of louping-ill virus does occur following PEG precipitation and if the aggregates persist throughout the vaccine production then it is probable that a proportion of the antigenic determinant sites will be effectively masked within the proteinaceous aggregates and the potency of resulting vaccines will therefore be reduced. This effect will also be exacerbated as the amount of protein precipitated from solution increases and may therefore account for the lower antibody responses produced by PEG vaccines prepared from virus grown in media containing serum compared to PEG vaccines prepared from virus grown in serum-free media.

The use of sonicated virus concentrates for subsequent vaccine production was not investigated and the adaptability of the PEG precipitation method for commercial scale vaccine production was not further pursued.

The lowest antibody titres were consistently demonstrated in animals inoculated with the experimental vaccines concentrated by methanol precipitation and the 1978-79 commercial vaccines. The results obtained from the 1978-79 commercial vaccines, prepared from virus antigen concentrate preparations that had been stored at -20^{o}C for 6 months or longer, also indicated that under such storage conditions the concentrate preparations lost antigenicity.

The responses evoked by vaccines prepared by concentrating by methanol precipitation, virus from both the methods of propagation described were not significantly different.

The technique of concentrating louping-ill virus antigen by ultrafiltration has been described previously (Wells and Reid, 1978). However in contrast to the studies reported here the inactivated virus, grown in serum-free media, was first concentrated 10-fold using a stirred cell ultrafiltration system and then extracted by acetone precipitation. The resultant vaccine was used solely to study the antibody response in newborn lambs. No attempts were made to omit the precipitation stage from the vaccine production schedule or to concentrate commercially produced virus.

That concentration of louping-ill virus by ultrafiltration was readily adaptable to a commercial scale of vaccine production was evident from the production of 12 litres of emulsified vaccine. The scale-up of the procedure from an experimental to a commercial level

was effective in terms of vaccine efficiency as measured by the serological responses evoked in sheep.

Louping-ill vaccine is designed primarily for the protection of sheep, however cattle are also susceptible to infection and require protection by vaccination. Vaccines prepared by ultrafiltration of virus harvests grown in media containing serum produced higher antibody titres in cattle than equivalent vaccines concentrated by methanol and the 1978-79 commercial vaccine. Antigen incorporated in oil adjuvant did not induce any signs of anaphylaxis but it is recognised that any future injections of lamb serum could be hazardous.

In conclusion therefore concentration of louping-ill virus by ultrafiltration produced vaccines of superior immunogenicity compared to corresponding vaccines concentrated by PEG and methanol precipitation. In addition vaccines concentrated 10- and 5-fold by ultrafiltration provoked a satisfactory immune response in sheep and cattle as defined in the British Pharmacopoeia (Veterinary) following a single 1ml dose in sheep and 2 1ml doses in cattle. In contrast, 2 1ml doses of the presently available commercial vaccine were required for sheep and 2 2ml doses for cattle.

Table 7.4 Antibody response in an inbred strain of mice (C57 black) and a random-bred strain of mice (Schneider Swiss White) to vaccine prepared during the commercial production of 1978-79.

Vaccine	Concentration Factor	Geometric mean reciprocal 42-day HAI titre	
		Schneider S.W.	C57 Black
PoolA*	10x	15.8	7.9
	2.5x	6.3	6.3
Pool B	10x	6.3	6.3
	2.5x	5.0	7.9
Pool C	10x	7.9	5.0
	2.5x	6.3	6.3
Pool D	10x	6.3	12.6
	2.5x	5.0	5.0
Pool E	10x	12.6	25
	2.5x	6.3	5.0
Pool F	10x	6.3	12.6
	2.5x	6.3	6.3

*Pre-blend potency vaccines (Appendix 3.1)

Antibody response following a double dose of vaccine

Table 7.5 Antibody response in Schneider Swiss White mice to vaccines prepared from virus propagated in serum-free media and concentrated by ultrafiltration (BAU concentrate vaccine)

*BAU Vaccine Concentration Factor	Number of mice having 21 day reciprocal HAI titre										Geometric mean reciprocal titre
	<10	10	20	40	80	160	320	640	1280	2560	
50x	1		1					1	2	5	630
10x			1			3		3		1	316
5x			2	2	1	3		1	1		126
2x	6	3	1								8
1x	6	1	1								6

Unconcentrated virus harvest: infectivity 1.2×10^8 pfu/ml HA 1/512

*Concentrated by stirred cell ultrafiltration

Antibody response following a single vaccination

Table 7.6 Antibody response in Schneider Swiss White mice to BAU concentrate vaccines: addition of vaccine supplements

Vaccine Supplement	Concentration Factor	Number of mice having 21-day reciprocal HAI titre										Mean reciprocal titre (log_{10})
		<10	10	20	40	80	160	320	640	1280	≥2560	
Formalinized lamb serum	10x					1					9	3.3 ± 0.16
Formalinized TPB	10x				1	1			1	2	5	3.1 ± 0.24
Formalinized LS + TPB	10x						2	4		1	3	2.9 ± 0.19
PBS + phenol	10x	1	1			1			2	1	4	2.7 ± 0.35
Methanol concentrated BHK cell extract	10x			2	1		2	3		1		2.1 ± 0.20
PBS + phenol	10x				1		2	3	1	2	1	2.6 ± 0.16

Unconcentrated virus harvest: infectivity 1.2×10^8 pfu/ml HA 1/512

Antibody response following a single vaccination

BAU concentrate: virus propagated in serum-free media, virus harvest concentrated by stirred cell ultrafiltration.

Mean ± s.e.

Table 7.7 Antibody response in Schneider Swiss White mice to vaccines prepared from virus propagated in serum-free media.

Vaccine	Concentration Factor	Number of mice having 21-day reciprocal HAI titre										Mean reciprocal titre (log_{10})
		<10	10	20	40	80	160	320	640	1280	≥2560	
BAU(SC)	10x				1		1			2	1	2.7 ± 0.33
BAP	10x			2	2	2	1					1.7 ± 0.12**
BAM	10x		1	2	1		1					1.5 ± 0.20**
BAU (SC)	5x	1				1			2	1		2.3 ± 0.40
BAP	5x	1		1	1	2						1.5 ± 0.20
BAM	5x	3			2							1.1 ± 0.21*
BAU (SC)	1x	3	2									0.8 ± 0.07
BAP	1x	1	6									1.0 ± 0.04
BAM	1.25x	5										0.7

Unconcentrated virus harvest: infectivity 1.2×10^8 pfu/ml HA 1/512

BAU(SC) virus propagated in serum-free media, concentrated by stirred cell ultrafiltration
BAP " " " " " " " " PEG precipitation
BAM " " " " " " " " methanol precipitation

The statistical difference between the titres produced by BAU concentrate vaccines and the BAP and BAM concentrate vaccines is indicated as follows: $*p < 0.05$ $**p < 0.01$

Mean ± s.e.

Table 7.8 Antibody response in Schneider Swiss White mice to vaccines prepared from virus propagated in BHK virus growth media.

Vaccine	Concentration Factor	Number of mice having 21-day reciprocal HAI titre										Mean reciprocal titre (log_{10})
		<10	10	20	40	80	160	320	640	1280	2560	
LSU(SC)	10x					1	1	1		1	1	2.6 ± 0.27
LSP	10x	3										0.7*
LSM	10x	2		1								0.8 ± 0.10*
LSU(SC)	5x	1		1	2	1						1.4 ± 0.20
LSP	5x	3										0.7*
LSM	5x	3										0.7*
LSU(SC)	1x	2	2		1							1.0 ± 0.16
LSP	1x	3										0.7
LSM	1x	3										0.7

Unconcentrated virus harvest: infectivity 4 x 10^8 pfu/ml HA 1/256

Antibody response following a single vaccination

LSU : virus harvest concentrated by stirred cell ultrafiltration
LSP : " " " " PEG precipitation
LSM : " " " " methanol precipitation

The statistical difference between the titres evoked by LSU concentrate vaccines and the LSP and LSM concentrate vaccines is indicated as follows *$p < 0.05$

Mean ± s.e.

Table 7.9 Geometric mean reciprocal HAI antibody titres in chickens injected with commercial vaccine and BAU concentrate vaccines

Vaccine	Concentration Factor	Days after primary vaccination 14	35
C2*	10x	5	8
C3	10x	5	10
D1	10x	6	40
D2	10x	5	20
D3	10x	6	13
Pool E	10x	6	16
Pool F	10x	13	13
BAU	50x	1995	19952
BAU	10x	398	1995
BAU	5x	50	1259
BAU	1x	10	25

*Pre-blend potency vaccines from commercial production 1978-79.

BAU: virus propagated in serum-free media; concentrated by stirred cell ultrafiltration

Revaccination at 14 days post primary vaccination

Table 7.10 Geometric mean reciprocal HAI antibody titres in sheep to final batches of commercial vaccine 1978-79

Vaccine	Period of storage of antigen concentrate at -20°C (months)	Days after primary vaccination	
		21*	49
Batch 1	6	10 2/6**	40 5/6
Batch 2	6	10 4/12	160 10/12
Batch 3	6	8 1/5	40 4/5
Batch 4	6	50 5/5	200 5/5
Batch 5	11	25 4/6	40 6/6
Batch 6	11	25 4/6	40 4/6
Batch 7	22	8 2/6	13 2/6
Batch 8	22	6 1/6	8 1/6

*Revaccinated with a further 1ml of vaccine

**Number of animals responding/number vaccinated

Table 7.11 Antibody response in sheep to BAU concentrate vaccines

BAU Vaccine Conc. Factor	No. of sheep having 28-day reciprocal HAI titre									Mean reciprocal 28-day titre (log_{10})	Mean reciprocal 126-day titre (log_{10})
	<10	10	20	40	80	160	320	640	1280		
10x					1	2			1	2.4 ± 0.25	2.4 ± 0.43
5x		1				1	1	1		2.1 ± 0.39	2.5 ± 0.32
2x	1		1	1	1					1.4 ± 0.25	2.0 ± 0.35
1x	3			1						0.9 ± 0.22	1.9 ± 0.30
0.5x	2	1	1							0.9 ± 0.14	1.7 ± 0.07

Unconcentrated virus harvest: Infectivity 1.2×10^8 pfu/ml HA 1/512

Antibody response following a single vaccination

BAU virus propagated in serum-free media, concentrated by stirred cell ultrafiltration

Mean ± s.e.

Table 7.12 Antibody response in sheep to vaccines prepared from virus propagated in BHK virus growth media

Vaccine	Concentration Factor	No. of sheep having 28-day reciprocal HAI titre								Mean reciprocal 28-day titre (log_{10})
		<10	10	20	40	80	160	320	640	
LSU(HF)	10x				2		1	2		2.1 ± 0.20
LSP	10x	1		1			1		1	1.8 ± 0.46
LSM	10x	1		1			3			1.7 ± 0.30
LSU(HF)	5x				1			3	1	2.4 ± 0.20
LSP	5x	2	1	1	1					1.1 ± 0.17
LSM	5x	1		1		1		1	1	1.8 ± 0.38
LSU(HF)	1x	2		1	1		1			1.3 ± 0.28
LSP	1x	1	1	3						1.1 ± 0.11
LSM	1x	4			1					0.9 ± 0.18

Unconcentrated virus harvest: infectivity 10^8 pfu/ml HA 1/128

Antibody response following a single vaccination

LSU(HF) virus harvest concentrated by hollow fibre ultrafiltration
LSP " " " " PEG precipitation
LSM " " " " methanol precipitation

Mean ± s.e.

Table 7.13 Antibody response in sheep to vaccine prepared from virus propagated in BHK virus growth media and concentrated using an Amicon hollow fibre filter*

Vaccine	Concentration Factor	Number of sheep having 28 day reciprocal HAI titre							Mean reciprocal 28-day titre (log_{10})	Mean reciprocal 126-day titre (log_{10})
		40	80	160	320	640	1280	2560		
Batch A	4.5x	1		2	1		1	1	2.5 $\pm$ 0.26	2.2 $\pm$ 0.34
Batch B	4.5x	1	1	3		1			2.2 $\pm$ 0.16	2.0 $\pm$ 0.23

Antibody response following a single vaccination

*Commercial scale of production

Mean $\pm$ s.e.

Table 7.14 Geometric mean reciprocal HAI antibody titres in cattle vaccinated with virus propagated in BHK virus growth media

Vaccine	Concentration Factor	Days after primary vaccination 28*	56	125
LSU(HF)	5x	10	501	100
		2/6**	6/6	6/6
	2.5x	13	158	100
		1/5	5/5	5/5
LSM	5x	5	6	16
		0/5	1/5	2/4
	2.5x	5	6	6
		0/5	2/5	2/5

Unconcentrated virus harvest: infectivity 10^8 pfu/ml

HA 1/128

*revaccinated with a further 1ml of vaccine

**Number of animals responding/number vaccinated

LSU(HF) virus harvest concentrated by hollow fibre ultrafiltration

LSM " " " " methanol precipitation

Table 7.15 Reciprocal HAI antibody titres in calves vaccinated with virus propagated in BHK virus growth media and concentrated by hollow fibre ultrafiltration

Virus Concentration Factor	Calf Number	Days after primary vaccination 28*	56	84
10x	**V516	5	160	160
	V683	80	1280	NA
	V684	10	160	NA
	X825	40	160	320
	X826	80	1280	2560
	Geometric mean	25	398	501
5x	**X843	20	80	80
	**X845	5	80	80
	**X846	5	160	160
	**X849	20	640	320
	Geometric mean	10	158	126

Unconcentrated virus harvest: infectivity 10^8 pfu/ml HA 1/128

*revaccinated with a further 1ml of vaccine

**animals hyperimmunized 176 days post primary vaccination

NA not available

CHAPTER EIGHT

THE STABILITY OF LOUPING-ILL-VIRUS VACCINES

INTRODUCTION

Traditionally the demand for louping-ill vaccine occurs in the early spring and late summer immediately preceding the periods of maximum tick activity.

The policy adopted for storage of vaccine is that directly following production, the 10-fold virus antigen concentrate (concentrated by methanol precipitation) is stored at -20°C. When a demand for vaccine is anticipated the concentrate is removed, diluted to the appropriate concentration as determined by the pre-blend potency test (Appendix 3.1), emulsified and, prior to commercial issue, subjected to final potency testing.

The minimum required storage life of emulsified vaccine is therefore approximately 6 months. As vaccine production presently operates on an annual or biannual basis the minimum required storage life of the virus antigen concentrate is 12 months.

The stability characteristics of the commercially produced 10-fold virus antigen concentrate and of the final emulsified vaccine preparation are undetermined. On numerous occasions, however, vaccine prepared from stored virus-antigen concentrate has failed to produce the expected antibody response as judged by the pre-blend potency test, and it has also been observed that the antigenic activity of concentrates (as reflected by the immunogenicity of the resulting vaccines) progressively diminishes as the period of storage at -20°C increases (Chapter Seven).

A requirement therefore exists for a comprehensive study of the stability of both antigen concentrate and emulsified vaccine.

The immunogenicity of experimental louping-ill vaccines prepared by PEG precipitation or ultrafiltration has been previously described (Chapter Seven). To further assess the efficacy of these experimental vaccines for commercial use, an evaluation of antigenic stability is also necessary.

Virus-antigen concentrates prepared by PEG precipitation and methanol precipitation contain phenol at a final concentration of 0.5 per cent (v/v), phenol is not however incorporated into the virus-antigen concentrates prepared by ultrafiltration. It would therefore also appear expedient to determine the effect, if any, of the presence of phenol on antigenic stability.

This chapter therefore examines the stability of commercially produced and experimentally produced virus-antigen concentrates and corresponding final vaccine preparations under defined storage conditions.

MATERIALS AND METHODS

PREPARATION OF VIRUS ANTIGEN CONCENTRATES AND EMULSIFIED VACCINE

Two louping-ill virus harvests (Batch 1 and 2), each 6 litres in volume, and a third harvest (Batch 3), 15 litres in volume, were prepared according to the method described in Chapter Four using Wheaton roller culture bottles and BHK virus growth media. Aliquots were removed from each of the 3 virus harvests and assayed for virus infectivity and HA activity. The harvests were then inactivated by treatment with 0.1 per cent formalin as described in Appendix 3.1.

Samples were removed from the inactivated virus suspensions and assayed for innocuity and sterility, as described in the commercial manufacturing protocol.

The 3 batches were each divided into 3 aliquots of equal volume and the aliquots concentrated 10-fold either by methanol (M) precipitation (as described in Appendix 3.1), PEG (P) precipitation or ultrafiltration (U). PEG precipitation was achieved using a 7 per cent (w/v) concentration of PEG 6000 and ultrafiltration was performed using an Amicon CH4 hollow fibre filter (Chapter Seven). Vaccines were prepared by emulsifying 80ml of each of the batch 1 and 2 virus antigen concentrates (1M, 1P, 1U, 2M, 2P and 2U) and 120ml of the batch 3 virus antigen concentrates (3M, 3P and 3U) with 160ml and 240ml respectively of Bayol F and Falba oil adjuvant using an Atomix laboratory blender. The vaccines, 9 in total, were dispensed in either 40ml or 20ml volumes in bottles fitted with rubber flanges and aluminium seals or in screw-capped bottles. The remaining concentrates were dispensed in 10ml volumes in screw-capped bottles.

STORAGE CONDITIONS

1. $-20^{o}C$.

Sixty ml of each of the batch 3 virus-antigen concentrates (3M, 3P and 3U) and 120ml of each of the corresponding emulsified vaccine preparations were stored at $-20^{o}C$ using a domestic type deep-freeze cabinet.

2. $4^{o}C$

One hundred and eighty ml of each of the batch 1, 2 and 3 virus-antigen concentrates (9 antigen concentrate preparations in total)

and 360ml of each of the corresponding emulsified preparations were stored in a 4°C cold room fitted with a continuous recording device.

3. 'Cool temp'

One hundred and twenty ml of each of the batch 1, 2 and 3 virus-antigen concentrates and 240ml of each of the resultant vaccine preparations were stored in an unheated room. The air temperature was recorded daily using a maximum-minimum thermometer. The mean temperature over a 24 week period was 17.6°C with a maximum recorded temperature of 26°C and a minimum temperature of 8°C.

4. 30°C

Sixty ml of each of the batch 1, 2 and 3 virus-antigen concentrates and 120ml of each of the corresponding emulsion preparations were incubated in a 30 ± 0.5°C waterbath. To determine the effect of phenol on the antigenic stability of concentrate preparations 75 µl were added to 15 ml of batch 3U virus antigen concentrate to give a final concentration of 0.5 per cent. An equal volume of PBS was added to a further 15ml volume of 3U antigen concentrate. The 2 concentrate preparations were also incubated at 30°C.

Aliquots of virus antigen concentrates and of emulsified vaccine preparations were removed after one and 2 weeks storage at 30°C and after 4, 8, 12 and 24 weeks storage at 4°C, 'cool temp' and -20°C.

EVALUATION OF VACCINE AND VIRUS-ANTIGEN CONCENTRATE STABILITY

Immediately prior to storage the immunogenicity of each of the 9 emulsified vaccine preparations was determined in mice. Immunogenicity of stored preparations was subsequently also evaluated in mice. After 24 weeks storage, selected emulsified vaccine

preparations and virus antigen concentrates were also assayed for potency in sheep.

1. Antibody response in mice

A 6ml volume of each of the emulsified vaccine preparations and a 5ml volume of each of the 10-fold virus antigen concentrates were removed from storage as required. Each virus-antigen concentrate was emulsified with oil adjuvant in a ratio of one part concentrate to 2 parts adjuvant. Potency of all vaccine preparations was evaluated by inoculating 3-week-old Swiss White mice i.p. with 0.5ml of vaccine. Each vaccine was injected into 8 mice. After 21 days 4 mice, selected at random from each group of 8, were killed and the blood collected by heart puncture. The remaining mice were then revaccinated with a further 0.5ml of the appropriate vaccine and 3 weeks later were killed and the blood collected. The blood was allowed to clot, the serum collected and tested for HAI antibody to louping-ill as described in Chapter Two. During the period between the initial vaccination and revaccination all vaccine preparations under test were stored at $4^{o}C$.

Mice inoculated with vaccine prepared from either 3U virus-antigen concentrate supplemented with 0.5 per cent (v/v) phenol or an equivalent phenol-free concentrate preparation received one injection of vaccine only.

2. Antibody response in sheep

Eighteen-month-old seronegative Greyface and Blackface sheep were used to assess the potency of vaccine preparations.

Phosphate buffered saline was emulsified with oil adjuvant in a ratio of one part PBS to 2 parts adjuvant. Equal volumes of 1U, 2U

and 3U emulsified vaccine preparations stored at either 4°C or at a 'cool temperature' for 24 weeks were pooled and added to an equal volume of emulsified PBS to give a 5-fold concentrate vaccine preparation. A volume of 3U emulsified vaccine stored at -20°C for 24 weeks was likewise diluted to a 5-fold concentrate vaccine. Emulsified vaccine preparations concentrated by methanol precipitation and stored at 4°C, 'cool' and -20°C were similarly sampled.

Aliquots (5ml) of 3U virus antigen concentrate were also removed after storage at -20°C and 4°C for 24 weeks and emulsified with oil adjuvant in a ratio one part concentrate to 2 parts adjuvant to give 10-fold concentrate vaccine preparations.

Sheep were injected s.c. behind the right shoulder with 1ml of vaccine and each vaccine was injected into 6 sheep. The animals were bled weekly for 4 weeks. The blood was allowed to clot, the serum collected and tested for HAI antibody activity.

Statistical analysis

For statistical analysis the data obtained from each of the 3 preparations concentrated by the same process (e.g. 1U, 2U, 3U) at each storage temperature were combined. Reciprocal HAI titres were transformed into logarithms to the base 10. Titres of <10 were deemed to have a logarithmic value of 0.7.

In instances where the rate of decline appeared to be a first-order-reaction standard statistical techniques were applied to calculate linear regressions and to assess the significance of the regression coefficients. The decrease in immunogenicity of several vaccine preparations concentrated by methanol or PEG precipitation did not follow first-order reaction kinetics. In such cases the significance

of decline was assessed using the 2-sample t-test. The 2-sample t-test was also used to determine the significance of the decrease in potency of vaccine preparations stored at 30°C for 2 weeks.

RESULTS

The mean 42-day HAI titres produced in mice by the pre-storage emulsified vaccine preparations concentrated by methanol precipitation, PEG precipitation and ultrafiltration were 1/79, 1/251 and 1/2511 respectively.

The effects of storage at refrigerated and room temperatures on the immunogenic stability of emulsified and non-emulsified virus antigen preparations concentrated by methanol precipitation, PEG precipitation and ultrafiltration are illustrated in Figs. 8.1 a-d.

THE STABILITY OF VIRUS ANTIGEN PREPARATIONS CONCENTRATED BY METHANOL PRECIPITATION

The stability characteristics, as determined by potency testing in mice, of emulsified and non-emulsified louping-ill virus-antigen concentrated by methanol precipitation are indicated in Table 8.1.

There was a significant decline ($p < 0.001$) in the immunogenicity of virus antigen preparations stored at 30°C for 2 weeks. The data were analysed using a 2-sample t-test.

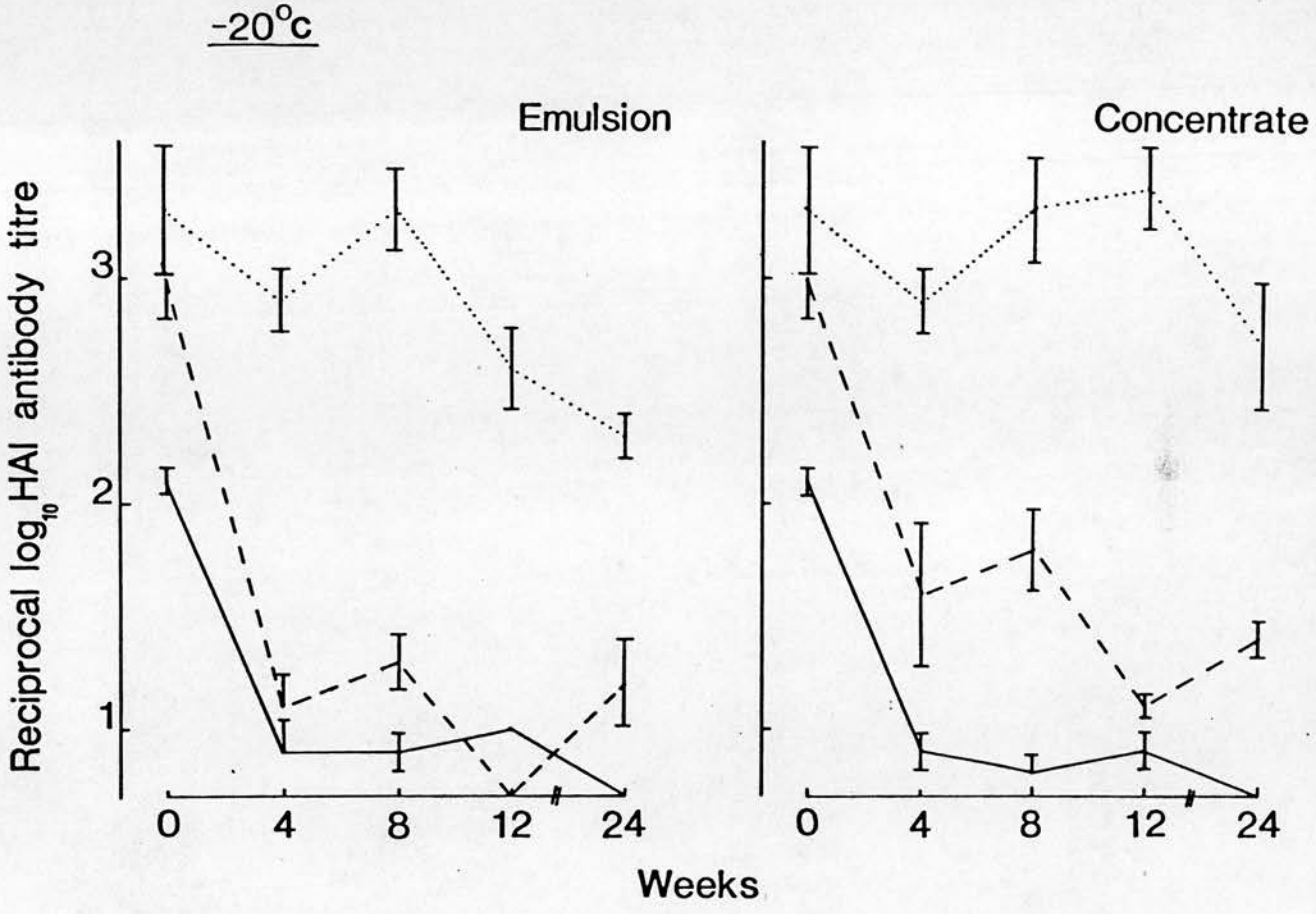
The immunogenicity of virus-antigen concentrate and emulsified vaccine significantly declined ($p < 0.001$) following storage at 'cool' or -20°C for one or 4 weeks respectively. A significant decline in the potency of emulsified vaccine held at 4°C for 4 weeks was also observed ($p < 0.001$). The data were analysed by a 2-sample t-test. The rate of decline in the immunogenicity of virus antigen

Fig. 8.1 a-d The stability characteristics of non-emulsified louping-ill virus antigen concentrates and emulsified final vaccines at refrigerated and room temperatures.

.... virus antigen concentrated by ultra-filtration

---- virus antigen concentrated by PEG precipitation

—— virus antigen concentrated by methanol precipitation


-20°c
Emulsion
Concentrate
Reciprocal log10 HAI antibody titre
3
2
1
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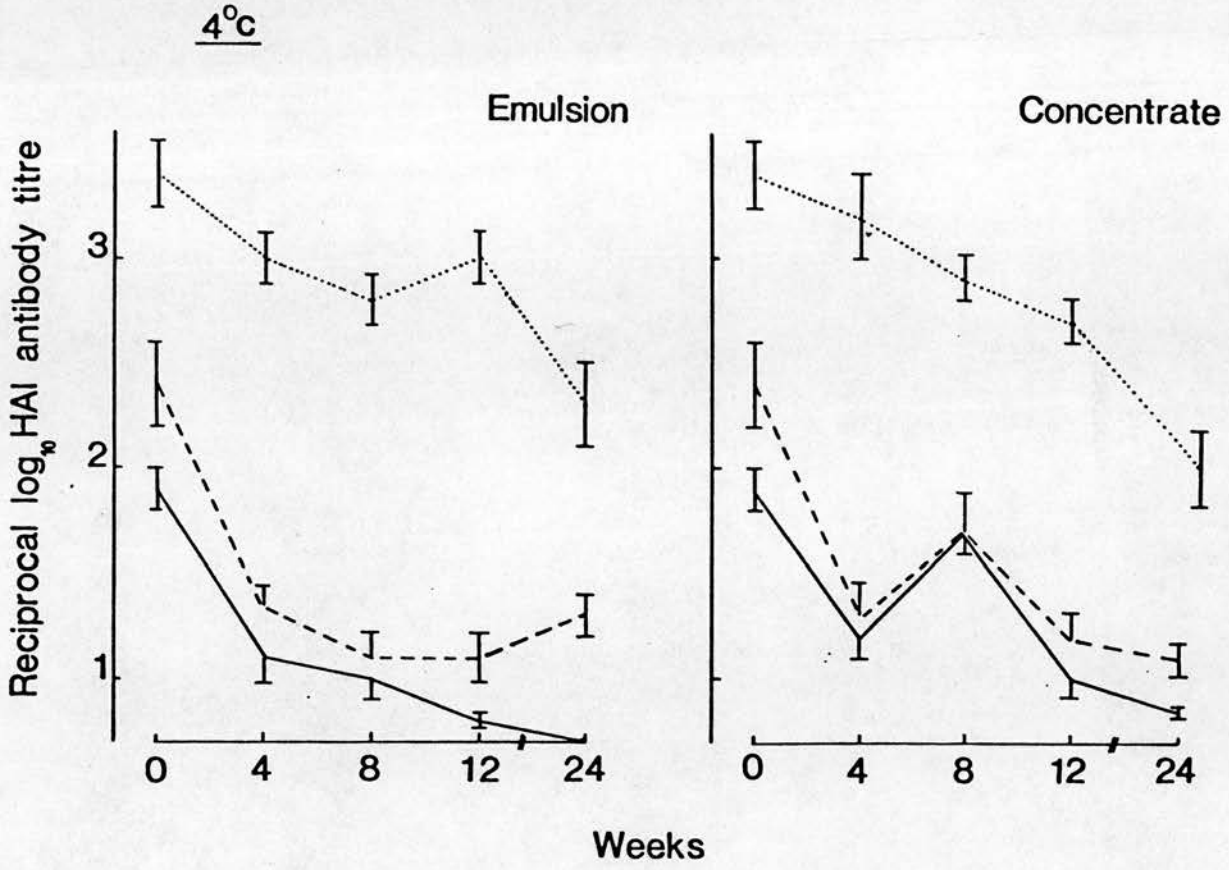
4
8
12
24
Weeks


4°c
Emulsion
Concentrate
Reciprocal log10 HAI antibody titre
3
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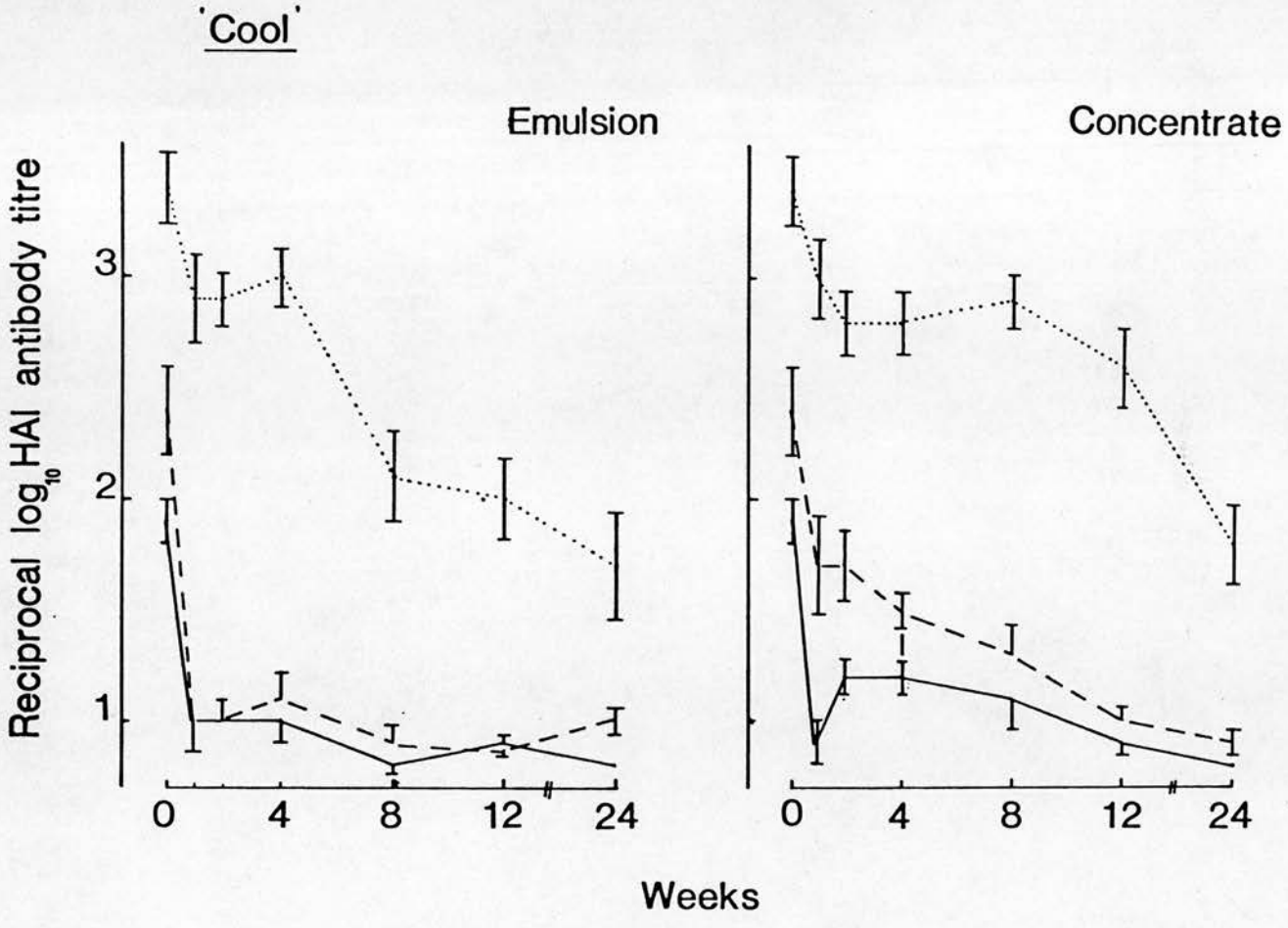
Weeks


'Cool'
Emulsion
Concentrate
Reciprocal log10 HAI antibody titre
3
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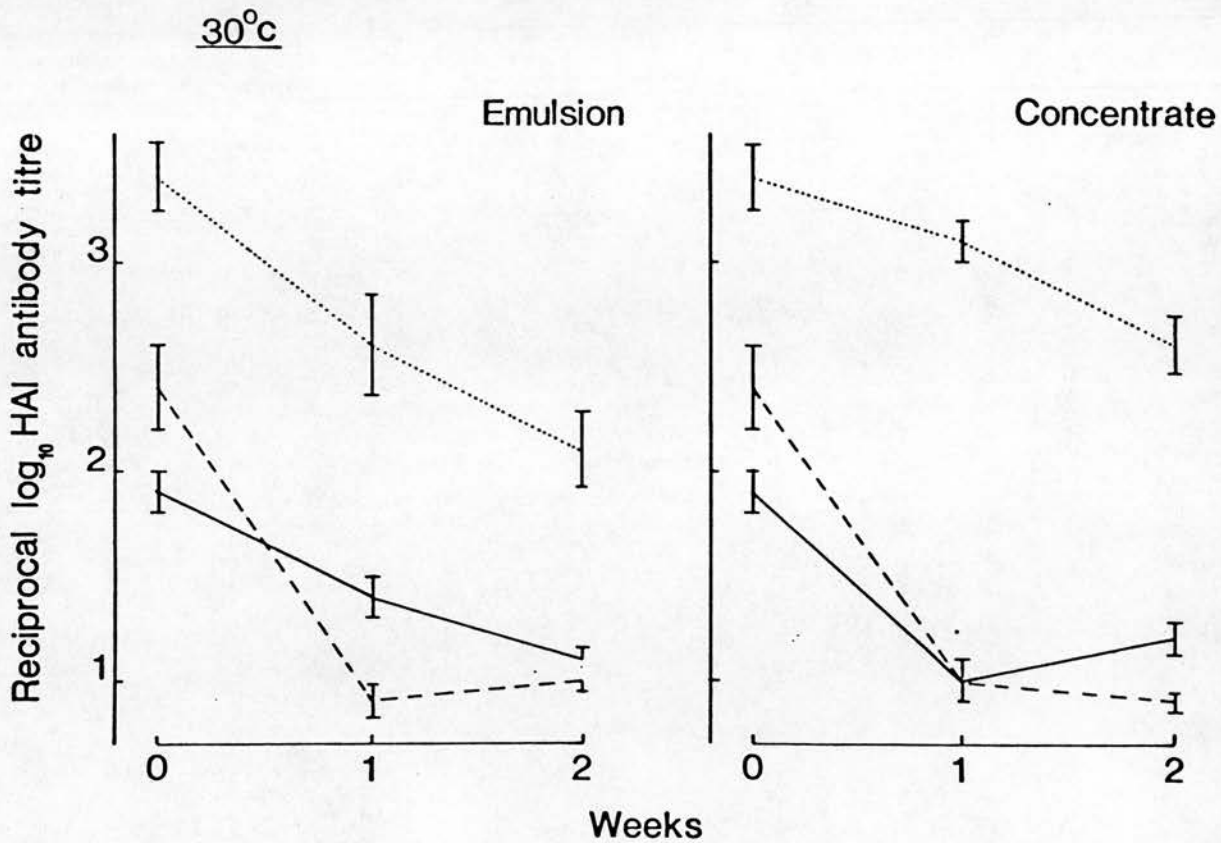
12
24
Weeks


30°c
Emulsion
Concentrate
Reciprocal log10 HAI antibody titre
3
2
1
0
1
2
0
1
2
Weeks

concentrate stored at 4°C for 6 months was deemed to be that of a first order reaction and the regression coefficient was -0.04 ($p < 0.001$).

The antibody responses in sheep to emulsified virus-antigen preparations previously stored at either 'cool', 4°C or -20°C for 6 months are given in Table 8.4. All animals vaccinated with emulsified virus-antigen stored at 'cool' were serologically non-responsive. Of the animals inoculated with emulsified virus-antigen stored at 4°C or -20°C, 17 per cent seroconverted with mean 28-day HAI titres of 1/10 and 1/8 respectively.

THE STABILITY OF VIRUS ANTIGEN PREPARATIONS CONCENTRATED BY PEG PRECIPITATION

The stability characteristics of emulsified and non-emulsified virus-antigen concentrated by PEG precipitation are shown in Table 8.2.

There was a significant decline ($p < 0.001$) in the potency of virus-antigen preparations stored at 30°C for 2 weeks. The immunogenicity of emulsified vaccines stored at 'cool' for one week or at 4°C and -20°C for 4 weeks also significantly decreased ($p < 0.001$, $p < 0.01$). The data were analysed by a 2-sample t-test. The inactivation curves obtained from virus-antigen concentrates stored at 'cool', 4°C or -20°C were assessed as being linear and the regression coefficients were -0.05 ($p < 0.001$) - 0.04 ($p < 0.001$) and -0.06 ($p < 0.01$) respectively.

Stored virus-antigen preparations concentrated by PEG precipitation were not potency tested in sheep.

THE STABILITY OF VIRUS-ANTIGEN PREPARATIONS CONCENTRATED BY ULTRAFILTRATION

The stability characteristics of emulsified and non-emulsified virus antigen concentrated by ultrafiltration are shown in Table 8.3.

There was a significant decline ($p < 0.001$) in the immunogenicity of virus-antigen preparations stored at 30°C for 2 weeks. The data were analysed using the 2-sample t-test.

The rate of decline in the immunogenicity of all other vaccine preparations followed first-order reaction kinetics. The regression coefficients are given in Table 8.3. The decline in vaccine potency over a 6 month storage period was significant ($p < 0.001$, $P < 0.01$) for all preparations except for virus antigen concentrate stored at -20°C ($p > 0.05$).

The antibody responses in sheep to emulsified virus-antigen preparations stored for 6 months at 'cool', 4°C and -20°C are given in Table 8.4. There was no detectable antibody response in animals vaccinated with emulsified virus antigen previously stored at 'cool'. Of the animals inoculated with emulsified virus-antigen preparations stored at either 4°C or -20°C, 50 per cent seroconverted with mean 28-day HAI titres of 1/10 and 1/13 respectively.

The antibody responses in sheep to vaccines prepared from non-emulsified virus antigen concentrate preparations previously stored at -20°C or 4°C for six months are also shown in Table 8.4. All animals inoculated with vaccine prepared from non-emulsified virus-antigen concentrate stored at -20°C seroconverted with a mean 28-day HAI titre of 1/398. Of the animals given vaccine derived from non-emulsified virus antigen stored at 4°C, 83 per cent were seropositive

and the mean 28-day HAI titre was 1/100.

The results are tabulated in full in Appendices 8.1-5.

THE EFFECT OF PHENOL ON THE STABILITY CHARACTERISTICS OF VIRUS ANTIGEN CONCENTRATES (NON-EMULSIFIED) PREPARED BY ULTRAFILTRATION

There was no significant difference in the antibody titres of mice injected with vaccines prepared from non-emulsified virus-antigen concentrates supplemented with either 0.5 per cent (v/v) PBS or 0.5 per cent (v/v) phenol and stored at 30°C for 14 days (Table 8.5). The data were analysed by the 2-sample t-test.

DISCUSSION

The stability characteristics of the commercial and experimentally prepared louping-ill virus vaccines stored at refrigerated and room temperatures for a period of up to 6 months were evaluated.

Potency tests in mice indicated that the immunogenicity of commercially produced virus-antigen concentrate and final emulsified vaccine rapidly declined to non-detectable levels within weeks of storage at both refrigerated and room temperatures. Potency tests in sheep further emphasised the antigenic instability of commercially produced emulsified final vaccine following storage for 6 months at refrigerated temperatures.

At present, the storage policy for commercial louping-ill vaccine concentrated by methanol precipitation is that bulk virus-antigen concentrate is maintained at -20°C and emulsified final vaccine at 4°C; the minimum required storage life of virus-antigen concentrate and final vaccine being 12 and 6 months respectively.

I found, however, that under these storage conditions commercially produced virus-antigen concentrate and final vaccine were antigenically unstable.

With respect to the storage characteristics of the experimentally produced virus-antigen concentrates and final vaccine preparations concentrated by PEG precipitation and ultrafiltration, potency testing both in mice and sheep indicated that only virus antigen prepared by ultrafiltration and stored as the concentrate at -20°C retained immunogenicity for a period of at least 6 months.

Although vaccine prepared from louping-ill virus antigen concentrated by ultrafiltration and stored as the concentrate at 4°C for 6 months also produced an acceptable antibody response in sheep, statistical analysis of the antibody response in mice did however indicate a significant decline in immunogenicity over this period.

Louping-ill virus antigen concentrated by ultrafiltration and emulsified with oil adjuvant was shown to be antigenically unstable following subsequent storage at refrigerated temperatures. Potency tests in mice indicated a significant decline in the immunogenicity of emulsified vaccine concentrated by ultrafiltration and stored for a period of 6 months at 4°C or -20°C, and the serological responses in sheep to such preparations also failed to comply with the louping-ill final vaccine potency test specifications.

All emulsified louping-ill vaccines, regardless of the method used to concentrate viral-antigen, and virus-antigen concentrates prepared either by methanol or PEG precipitation, contain phenol at a final concentration of 0.5 per cent (v/v). Only virus antigen

concentrate prepared by ultrafiltration is phenol-free and of all the vaccine preparations studied only this virus-antigen concentrate was antigenically stable over a prolonged storage period. An attempt to determine whether phenol exerts an adverse effect on the stability of louping-ill virus antigen failed. The conditions under which the investigation was performed, however, were limited both with respect to the temperature of storage and the duration of storage and a further more comprehensive study is required.

A review of the literature indicated that information relating to the storage characteristics of inactivated togavirus vaccines, other than louping-ill-virus vaccine, is negligible. The stability characteristics of the only established live togavirus vaccine, yellow fever vaccine, are however well documented (Burfoot, Young and Finter, 1977).

To conclude, therefore, both the commercially produced virus-antigen concentrate and the commercial emulsified vaccine were shown to be antigenically unstable following storage at refrigerated and room temperatures. These findings further emphasise the inadequacy of this method of vaccine production.

Although virus-antigen concentrated by ultrafiltration and stored as concentrate at -20^{o}C retained antigenicity, emulsification of the concentrate and subsequent storage at refrigerated temperatures resulted in loss of immunogenicity. The implications of the instability of emulsified vaccine prepared from louping-ill virus-antigen concentrated by ultrafiltration with regard to the use of this concentration technique for commercial production of vaccine is discussed further in Chapter Nine.

Table 8.1 The stability characteristics of emulsified and non-emulsified louping-ill virus-antigen concentrated by methanol precipitation

	Storage Temp °C	Geometric mean reciprocal 42-day HAI titre in mice (log_{10}) Weeks of storage 0	1	2	4	8	12	24	Regression* coefficient	Significance of decline
Emulsion	30	1.9	1.4	1.1					-	$p < 0.001$
Concentrate	30	1.9	1.0	1.2					-	$p < 0.001$
Emulsion	'Cool'	1.9	1.0	1.0	1.0	0.8	0.9	0.8	-	$p < 0.001$
Concentrate	'Cool'	1.9	0.9	1.2	1.2	1.1	0.9	0.8	-	$p < 0.001$
Emulsion	4	1.9	NT	NT	1.1	1.0	0.8	0.7	-	$p < 0.001$
Concentrate	4	1.9	NT	NT	1.2	1.7	1.0	0.9	- 0.04	$p < 0.001$
Emulsion	-20	2.1	NT	NT	0.9	0.9	1.0	0.7	-	$p < 0.001$
Concentrate	-20	2.1	NT	NT	0.9	0.8	0.9	0.7	-	$p < 0.001$

NT not tested

*Where no regression coefficient is given the rate of decline was deemed not to be a first-order reaction, the significance of decline was therefore assessed by comparing the difference between the pre-storage antibody titres and those obtained after either one week of storage ('cool'), 2 weeks of storage (30°C), or 4 weeks of storage (-20°C, 4°C).

Table 8.2 The stability characteristics of emulsified and non-emulsified louping-ill virus-antigen concentrated by polyethylene glycol precipitation

	Storage Temp °C	Geometric mean reciprocal 42-day HAI titre in mice (log_{10}) Weeks of storage 0	1	2	4	8	12	24	Regression* coefficient	Significance of decline
Emulsion	30	2.4	0.9	1.0					-	$p < 0.001$
Concentrate	30	2.4	1.0	0.9					-	$p < 0.001$
Emulsion	'Cool'	2.4	1.0	0.9	1.1	0.8	0.9	1.0	-	$p < 0.001$
Concentrate	'Cool'	2.4	1.7	1.7	1.5	1.3	1.0	0.9	-0.05	$p < 0.001$
Emulsion	4	2.4	NT	NT	1.3	1.1	1.1	1.3	-	$p < 0.01$
Concentrate	4	2.4	NT	NT	1.3	1.7	1.2	1.1	-0.04	$p < 0.001$
Emulsion	-20	3.0	NT	NT	1.1	1.3	0.7	1.2	-	$p < 0.01$
Concentrate	-20	3.0	NT	NT	1.6	1.8	1.1	1.4	-0.06	$p < 0.01$

NT not tested

*Where no regression coefficient is given the rate of decline was deemed not to be a first-order reaction, the significance of decline was therefore assessed by comparing the difference between the pre-storage antibody titres and those obtained after either one week of storage ('cool'), 2 weeks of storage (30°C) or 4 weeks of storage (-20°C, 4°C).

Table 8.3 The stability characteristics of emulsified and non-emulsified louping-ill virus-antigen concentrated by ultrafiltration

	Storage Temp °C	Geometric mean reciprocal 42-day HAI titre in mice (log_{10}) Weeks of storage 0	1	2	4	8	12	24	Regression* coefficient	Significance of decline
Emulsion	30	3.4	2.6	2.1					-	$p < 0.001$
Concentrate	30	3.4	3.1	2.6					-	$p < 0.001$
Emulsion	'Cool'	3.4	2.9	2.9	3.0	2.1	2.0	1.7	-0.07	$p < 0.001$
Concentrate	'Cool'	3.4	3.0	2.8	2.9	2.8	2.8	1.8	-0.05	$p < 0.001$
Emulsion	4	3.4	NT	NT	3.0	2.8	3.0	2.3	-0.04	$p < 0.001$
Concentrate	4	3.4	NT	NT	3.2	2.9	2.7	2.0	-0.06	$p < 0.001$
Emulsion	-20	3.3	NT	NT	2.9	3.3	2.6	2.3	-0.04	$p < 0.01$
Concentrate	-20	3.3	NT	NT	2.9	3.3	3.4	2.7	-0.02	$p > 0.05$

NT not tested

*Where no regression coefficient is given the rate of decline was deemed not to be a first-order reaction, the significance of decline was therefore assessed by comparing the difference between the pre-storage antibody titres and those obtained after 2 weeks of storage.

Table 8.4 Antibody response in sheep to vaccines stored for 6 months, either as the 10-fold virus-antigen concentrate or as the 10-fold emulsified vaccine

Vaccine	Storage conditions		No. of sheep having 28-day reciprocal HAI titre									Geometric mean reciprocal titre
	Preparation	Temp	<10	10	20	40	80	160	320	640	1280	
U	Emulsion	-20°C	3		2		1					13
M	Emulsion	-20°C	5			1						8
U	Emulsion	4°C	3	2		1						10
M	Emulsion	4°C	5					1				10
U	Emulsion	'Cool'	6									5
M	Emulsion	'Cool'	6									5
U	Concentrate	-20°C				1			3		2	398
U	Concentrate	4°C	1		1			1	2	1		100

U: concentrated by ultrafiltration
M: concentrated by methanol precipitation

Stored 10-fold emulsified vaccine preparations diluted with equal volumes of emulsified PBS and injected into sheep as 5-fold concentrated vaccines.

Stored 10-fold virus antigen concentrates emulsified and injected into sheep as 10-fold concentrated vaccines.

Table 8.5 The effect of phenol on the stability of virus-antigen concentrate prepared by ultrafiltration

Vaccine	Storage Conditions	Geometric mean reciprocal HAI titre after weeks of storage		
		0	1	2
3U	10x concentrate + 0.5% v/v PBS at 30°C	63	40	40
3U	10x concentrate + 0.5% v/v phenol at 30°C	50	32	25

CHAPTER NINE

GENERAL DISCUSSION

Louping-ill is probably the most important virus infection of sheep in Scotland and Northern England and recent appearance of the disease in areas where it had hitherto been unrecognised, together with the increasing demand for vaccine, emphasises the need for effective prophylactic control.

Control of arthropod-transmitted viral disease can be effected by 2 basic methods; control of the vector or the immunization of the susceptible host population through large-scale vaccination. In the majority of instances control by the latter method is most practical and vaccines have been, or are in the process of being, developed (Table 9.1).

The animals most susceptible to louping-ill infection are (i) hoggs returning from overwintering to tick-infected pasture, (ii) purchased animals, and (iii) lambs (Pool, Brownlee and Wilson, 1930). Losses may also be experienced in all ages of sheep when louping-ill is first encountered on a farm (C. Bannatyne, personal communication). Immunization of susceptible animals is presently effected using a formalin-inactivated, methanol-precipitated, oil adjuvant vaccine derived from virus infected BHK-21 culture.

Production of this vaccine is based on the methodology developed by Brotherston and Boyce (1970) in the experimental preparation of highly immunogenic louping-ill vaccine propagated in primary sheep kidney cell culture. A single dose of this vaccine was shown to be sufficient to provoke a satisfactory immune response in sheep.

In contrast however the immunogenicity of the presently available vaccine, based on propagation of virus in BHK-21 cells, is low and often considerably variable and 2 doses are required to produce a satisfactory immune response in sheep. Furthermore it has also been observed that even following the recommended 2 dose schedule a number of animals persistently remain seronegative or seroconvert to minimal HAI antibody titres only.

Studies undertaken by the author and reported here demonstrated that following experimental challenge animals immunized with this vaccine were protected against clinical disease. Circulation of virus was nevertheless demonstrated in those animals exhibiting minimal or undetectable levels of specific humoral antibody. The intensity of viraemia was however insufficient to cause clinical disease and below the threshold titre required to establish virus in the tick. It is evident therefore that despite the absence of a detectable serological response, the immune system of such animals is nevertheless sufficiently sensitized to protect against clinical disease following experimental challenge.

Under field conditions however vaccination may not always induce an optimal immune response; concurrent infection of mice (Buxton, Reid and Pow, 1979) and sheep (Reid, Buxton, Pow and Finlayson, in press) with Toxoplasma gondii has been shown to reduce the antibody response to louping-ill vaccine and studies with tick-borne fever (TBF) have shown that infected sheep have depressed immune responses to para-influenza-3 virus (M. Batungbacal, personal communication). Animals concurrently infected with TBF may therefore be also less

able to respond to louping-ill vaccine. It is possible therefore that under certain conditions the immune responsiveness in a proportion of sheep will be suboptimal and following immunization with low potency vaccine the response may be insufficient to protect the animals from clinical disease following natural challenge. In addition animals immunized with a low potency vaccine may therefore develop viraemias of sufficient intensity to reinfect the tick vector and transmission and maintenance of the virus in nature will be unaffected by vaccination.

The initial protection of lambs born at or around the peak period of the spring phase of tick activity depends entirely upon maternal antibody acquired from colostrum within the first 36 hours of birth (Brambell, 1958). Reid and Boyce (1976) demonstrated that lambs with titres of maternal antibody in excess of 1/40 are refractory to infection and protection persists for at least 3 weeks following the decline of maternal HAI antibody to undetectable levels. Experimentally the titres of louping-ill HAI antibodies in the sera of lambs receiving colostral antibodies from immune ewes were shown to parallel the titres of the dams (Williams and Thorburn, 1961; Brotherston, Bannatyne, Mathieson and Nicholson, 1971). Under field conditions however transfer of antibody may not be as efficient.

Thus, at lambing, ewes vaccinated 3 or 15 months previously with vaccine of low potency may, unless exposed to natural infection in the intervening period, exhibit only minimal levels of specific antibody. Transfer of maternal immunity to the lamb will therefore also be minimal and, as the half-life of colostrum-derived HAI antibody is 13.5 days (Brotherston - cited by Reid, PhD Thesis, 1975), such

animals may therefore become susceptible to infection within weeks of birth and prior to the cessation of the spring/summer period of tick activity.

Similarities between the present vaccine and the inactivated brain tissue vaccine that was available up until the 1960s are evident; the immunogenicity of the brain tissue vaccine was also low and a single dose likewise failed to induce a serological response. In addition the apparent priming of the immune system in the absence of a detectable serological response, as evident from the vaccination studies reported here, is comparable to the suggested mode of action of the brain tissue vaccine (Gordon, 1934; Gordon, Brownlee, Wilson and MacLeod, 1962; Edward, 1947b; Smith McMahon, O'Reilly, Wilson and Robertson, 1964; Smith, 1969; Brotherston, Bannatyne, Mathieson and Nicolson, 1971).

During the period when this brain tissue vaccine was the only method of prophylaxis a considerable mortality in lambs due to louping-ill was reported on 2 separate occasions (Gordon, 1934; Smith, McMahon, O'Reilly, Wilson and Robertson, 1964) and Smith et al (1964) expressed concern over the use of a low potency vaccine which, although preventing clinical infection in ewes may however fail to provide a substantial maternal immunity in lambs.

It is evident therefore that for effective and practical prophylactic control of louping-ill within the farming industry the use of a vaccine capable of eliciting the highest possible serological response following a single inoculation is particularly desirable. There is also evidence to suggest that, under the recommended storage conditions, the commercial vaccine is immunologically unstable. Stability studies have confirmed this observation as both virus

antigen concentrate and emulsified vaccine were immunologically unstable following storage at refrigerated temperatures for periods of less than 6 months.

The principal aim of this project was therefore to develop a one dose vaccine antigenically stable and capable of consistently producing a satisfactory immune response in sheep.

Optimal conditions for the growth of louping-ill virus in BHK-21 cell monolayer cultures were determined experimentally and, using this technique under commercial conditions, titres of individual harvests were found to range from 6.1 $\log_{10}$ pfu/ml to 8.5 $\log_{10}$ pfu/ml. A direct correlation between the pre-inactivation infectivity titres of virus suspensions and the immunogenicity of the resulting vaccines was demonstrated and a threshold titre of 8.0 $\log_{10}$ pfu/ml determined; harvests with titres below this produced unacceptable vaccines. The immunogenicity of louping-ill vaccine is thus dependent on the pre-inactivation infectivity titre of the virus harvest.

The titre of louping-ill virus propagated in secondary monolayers of sheep kidney cells was 10^{-7} mouse LD_{50}/ml (Brotherston and Boyce, 1970), and vaccines prepared from these inactivated unconcentrated virus harvests were likewise shown to be non-immunogenic.

That vaccine potency is determined by the antigenic mass of the source virus suspension has been well illustrated for other members of the Togaviridae (Levkovich, 1962; Smorodintsev and Ilyenko, 1962; Crawford, Dayhuff and White, 1968), and Darwish and Hammon (1966b), developing an inactivated Japanese B encephalitis virus vaccine derived from hamster kidney cell culture, also demonstrated a direct correlation between the pre-inactivation infectivity titre of the source virus suspension and vaccine immunogenicity.

The failure to produce highly immunogenic vaccine against the alphavirus, Chikungunya virus, from chick embryo monolayer cultures (Harrison, Binn and Randall, 1967) was partially attributed to low pre-inactivation infectivity titres. White, Berman and Lowenthal (1972), using chick embryo suspension cultures, however, propagated virus to considerably higher titres and subsequently produced highly potent vaccines.

The growth of louping-ill virus in other cell culture systems was not examined, although recently Malewicz and Jenkin (1979) propagated Dengue virus type 2 in BHK-21 cells adapted to shaker culture to higher infectivity titres than had been previously reported in other cell systems. It is possible therefore that a similar technique adapted for the growth of louping-ill virus may also significantly increase virus yields.

Using the present method of propagation the infectivity titre of the commercially produced louping-ill virus harvests will approximate to the threshold titre of 8.0 $\log_{10}$ pfu/ml. Concentration of louping-ill viral antigen is thus a particularly necessary and important stage in the production of potent vaccine.

Production of the presently available vaccine involves the concentration of viral antigen by methanol precipitation. The studies described here however demonstrate that this method of concentrating commercially produced virus antigen was ineffective. There was no increase in the immunogenicity of concentrated virus harvests compared to unconcentrated harvests and in several instances the concentration procedure actually resulted in vaccines of decreased potency.

In contrast Brotherston and Boyce (1970) reported that methanol concentration of louping-ill virus antigen derived from sheep kidney cells was particularly effective and produced highly potent vaccines. Vaccine immunogenicity could not however be wholly attributed to the concentration process as dilution of the concentrates to volumes in excess of the original volume still produced immunogenic vaccine. It was suggested therefore that in addition to concentrating viral antigen, methanol precipitation also enhanced surface antigenicity by releasing or 'unmasking' hidden antigen.

The efficacy of this method of concentrating louping-ill virus antigen is thus apparently dependent on the method of virus propagation, concentration is effective with virus derived from homologous cell culture (sheep kidney cells) but ineffective when applied to virus propagated in a heterologous cell culture system (BHK-21 cells).

In an attempt to offer an explanation for this discrepancy between the results reported by Brotherston and Boyce (1970) and the work described here, a review of the current knowledge relating to the surface morphology and antigenicity of the Togaviridae is necessary.

Togaviruses are small RNA viruses constructed of a spherical nucleocapsid and surrounded by an outer lipoprotein envelope carrying glycoprotein projections on its surface. Togavirus morphogenesis is entirely cytoplasmic although alphaviruses and flaviviruses differ with respect to viral maturation. The envelope of the alphaviruses is acquired as the nucleocapsid buds through

the marginal membrane, whereas flaviviruses emerge from internal vacuolar membranes (Horzinek, 1973).

Analysis of the structural proteins of the togaviruses by polyacrylamide gel electrophoresis has revealed 2 glycoproteins in the viral envelope (Schlesinger, Schlesinger and Burge, 1972; Garoff, Simons and Renkonen, 1974). That the envelope glycoproteins are the principal determinants of HA activity and surface antigenicity has been well illustrated (Mussgay, Weiland, Weiland and Härtner, 1976; Kennedy, 1974; Appleyard, Oram and Stanley, 1970; Bose and Sagik, 1970; Pedersen and Eddy, 1974; Pederson, Slocum and Eddy, 1973; Heinz and Kunz, 1977b; Kitano, Suzuki and Yamaguchi, 1974). Kitano, Suzuki and Yamaguchi (1974) further demonstrated that antigenicity is specifically associated with the glycoproteins of the surface projections of Japanese encephalitis virus, although activity could not be definitively assigned to either the sugar or polypeptide component of the glycoprotein. Mussgay, Weiland, Weiland and Härtner (1976) and Kennedy (1974), studying the alphavirus, Semliki Forest virus, suggested that it is the polypeptide portion rather than the carbohydrate residues that determine surface antigenicity and haemagglutination activity and that the integrity of this component is essential for expression of these properties.

Carbohydrate is a universal constituent of the lipoprotein membrane of all classes of enveloped viruses and is thought to be associated with the membrane protein of the viral envelope (Strauss, Burge and Darnell, 1970). Burge and Huang (1970) working with a togavirus (Sindbis virus) and a rhabdovirus (vesicular stomatitis virus suggested that the carbohydrate moiety of the envelope glycoprotein

may be specified principally by the host cell.

X-ray diffraction studies of Sindbis virus (Harrison, David, Jumblatt and Darnell, 1971) indicated that the lipids in the lipoprotein envelope are arranged as a bilayer. The polar groups orientate towards the centre of the virus whilst the outer groups are associated with the peripheral envelope glycoprotein. The suggestion that at least part of the glycoprotein surface projections pass through the lipid bilayer was proposed by Brown, Smale and Horzinek (1974); Utermann and Simons (1974); Garoff (1974); and Garoff and Simons (1974).

Whether the lipid component is host cell determined or a reflection of the lipid affinities of the viral envelope polypeptide is not established. David (1971), studying the lipid patterns of Sindbis virus grown in either chick embryo fibroblasts or BHK-21 cells, proposed that the lipid composition is determined by the viral protein. Conversely, Heydrick, Comer and Wachter (1971) demonstrated that Venezuelan equine encephalitis virus propagated in L cells of mouse origin had a lipid composition different from virus grown in chick embryo culture. Laine, Kettunen, Gahmberg, Käriäinen and Renkonen (1972) also observed that the fatty acids of the different lipid classes present in Semliki Forest virus and host BHK-21 plasma membranes were similar. Furthermore when the phospholipids of members of 3 different groups of viruses (toga, paramyxo and leukoviruses) grown in the same host cell species were compared, similar patterns were observed (Quigley, Rifkin and Reich, 1971).

If the lipid and carbohydrate composition of the viral envelope is specified by the host cell, it may not be unreasonable to assume that louping-ill virus propagated in different cell systems may also differ in the chemical make-up of both the lipid and carbohydrate constituents of the virus envelope.

A dissimilarity in the lipid composition of Venezuelan equine encephalitis virus propagated in L cells or chick embryo cell culture was also reflected by a difference in the sensitivity of virus to thermal inactivation (Heydrick, Comer and Wachter, 1971); it is possible therefore that further biochemical characteristics may also be modified by an alteration in lipid composition.

Louping-ill virus propagated in sheep kidney cell culture and BHK-21 cell culture may therefore not only differ with respect to lipid and carbohydrate composition but also in the sensitivity to treatment with organic solvents.

That methanol is capable of modifying the surface spatial arrangement of enveloped viruses has been demonstrated (Jensen, Kemeny and Stone, 1979). They observed that following methanol treatment, a loss of peplomers from the surface of the virus of transmissible gastroenteritis of pigs, a member of the coronaviridae, was apparent and it was suggested that this was due to an interaction between methanol and the lipid component of the virus envelope. That such an alteration in surface morphology was adversely associated with immunogenicity of the virus was demonstrated by Garwes and Pocock (1975).

It is therefore possible that methanol treatment of louping-ill virus may also produce distinct morphological changes in the antigenic

determinant sites according to the composition of the lipid bilayer and reflected in the surface antigenic activity. As proposed by Brotherston and Boyce (1970), methanol precipitation of virus propagated in sheep kidney cell culture may therefore generate modifications in the surface structure that subsequently augment antigenicity and vaccine potency. In contrast, however, it is suggested that methanol treatment of louping-ill virus derived from BHK-21 cell culture may, as a consequence of a variance in lipid composition, produce changes in the surface morphology that adversely affect antigenic activity resulting in vaccines of inferior immunogenicity.

Burge and Strauss (1970) and Strauss, Burge and Darnell (1970) studying the alphavirus, Sindbis virus, have evidence to suggest that all viral carbohydrate is covalently bound to the virus specified envelope protein although the function of the carbohydrate remains unknown. If, as suggested by Burge and Huang (1970), the virus carbohydrate is determined by host cell enzymes then the glycoprotein may therefore represent a host modification of a virus determined protein and behave as a hybrid antigen, part host specified and part virus specified. Although the immunological consequences of such a situation are not obvious the use of a heterologous rather than a homologous cell culture system for propagating virus required for subsequent vaccine production may therefore adversely affect the specific antigenic activity determined by the surface glycoproteins and thus the immunogenicity of resulting vaccine preparations.

The low potency characteristic of the presently available louping-ill vaccine may therefore be attributable to:

(1) the use of a heterologous cell culture system for virus propagation.

(2) the concentration of viral antigen by the method of methanol precipitation

(3) vaccine instability

and a re-evaluation of each of these features of vaccine production is therefore paramount to the production of a louping-ill vaccine of improved quality.

As the use of homologous primary cell cultures is not feasible for the large-scale production of commercial vaccine and a homologous cell line is unavailable, the use of BHK-21 cells for culturing virus inevitably remains an integral feature of vaccine production.

Louping-ill virus antigen prepared from infected BHK-21 cell cultures was successfully and efficiently concentrated by the technique of ultrafiltration. The potencies of resulting vaccines prepared under both experimental and commercial conditions of production were consistently higher than corresponding vaccines prepared by concentrating viral antigen by methanol precipitation. In addition, the immunogenicity of vaccines concentrated either 10- or 5-fold by ultrafiltration provoked a satisfactory immune response in sheep and cattle in compliance with the potency test specification following a single 1ml dose and 2 1ml doses respectively.

Ultrafiltration is a purely physical method of concentration, essentially approximating to the simple process of de-watering and removal of low molecular weight salts. It is thus a particularly mild method of concentrating viral antigen and it is probably this characteristic of the technique that allows for effective

concentration and recovery of viral antigen and the subsequent production of highly potent vaccines.

The indiscriminant nature of the concentration process may however be a potential criticism of this technique as in addition to the concentration of specific viral antigen, non-viral proteins derived from the cell substrate and media constituents of an equivalent or higher molecular weight will also be simultaneously concentrated. Antigenically therefore the resulting concentrate may be particularly heterogenous in character. Inhibition of, or interference with the immune response to specific antigen by non-specific protein was not however evident in the studies reported here.

The advantages of concentrating commercially prepared louping-ill virus antigen by ultrafiltration as opposed to methanol precipitation are numerous: from the farmers' and veterinary practitioners' point of view a single dose vaccine is preferable to a double dose vaccine, both with respect to the practical and managerial aspects of vaccination and the vaccine purchasing costs. In addition the serological responses evoked by vaccines concentrated by ultrafiltration have been sufficiently high to suggest that:
(1) vaccinated ewes will transfer a substantial maternal antibody to the lamb
(2) in those animals failing to produce an optimal serological response to vaccination, the level of circulating antibody evoked will nevertheless be sufficient to protect against clinical infection, and
(3) replication of virus in vaccinated animals following natural exposure to virus will be minimal and less than the infection threshold titre in ticks. Systematic immunization using a vaccine concentrated

by ultrafiltration will therefore abrogate virus transmission to the tick and be instrumental in reducing the natural reservoir of virus.

From the manufacturer's point of view ultrafiltration is unequivocably a more favourable method both in terms of simplicity of vaccine production, overall manufacturing costs and safety.

The technique is simple in operation and, in contrast to the methanol method, demands on manpower resources are minimal. Expenditure is limited to the initial purchase of an appropriate hollow fibre ultrafiltration system and as the hollow fibre cartridges are reusable over an extended period without loss of filtration efficiency, further costs are negligible.

Commercial vaccine production involves the use of large volumes of inflammable solvent thus constituting a serious fire hazard. In contrast the technique of ultrafiltration can be used without risk.

The preparation of louping-ill vaccine using this technique of ultrafiltration to concentrate viral antigen appears therefore to satisfy the demands cited earlier, i.e. the development of a highly immunogenic vaccine capable of eliciting a satisfactory immune response in sheep following a single dose.

With respect to the stability of such vaccines, assessment of the storage characteristics of the virus antigen concentrate is necessarily an on-going study. However, potency testing in sheep has so far indicated that the concentrate retains antigenicity for at least 6 months stored at either $4^{o}C$ or $-20^{o}C$. In contrast however storage of the emulsified vaccine for a period of 6 months at either $4^{o}C$ or $-20^{o}C$ resulted in almost total loss of immunogenicity. As the minimum required storage life of emulsified vaccine is 6 months,

it is apparent therefore that the storage characteristics of the emulsified vaccine under such conditions are inadequate.

Equivalent vaccine preparations concentrated by PEG precipitation were also subjected to stability studies and both the concentrate and the emulsified vaccine were found to be non-immunogenic when tested in mice or sheep after 6 months storage at $4^{o}C$ or $-20^{o}C$. Commercially produced virus antigen concentrate, prepared by methanol precipitation and the resulting emulsified vaccine were similarly non-immunogenic following equivalent stability tests.

Thus of all the vaccine preparations examined only virus antigen concentrate prepared by ultrafiltration retained potency following prolonged storage periods. A distinctive feature of this preparation was the absence of phenol. Virus antigen precipitated by methanol and PEG were routinely resuspended in PBS which, for bactericidal purposes, was supplemented with 0.5 per cent (v/v) phenol and all antigen concentrates were emulsified with oil adjuvant also supplemented with an equivalent concentration of phenol.

Attempts to determine whether exposure to phenol produced an adverse effect on vaccine immunogenicity were inconclusive. Nevertheless the possibility that during prolonged storage the continuous exposure of antigen to phenol or the oxidation products of phenol may be detrimental to antigenic activity cannot be excluded.

It is of interest to note that in the production of highly potent louping-ill vaccine derived from primary sheep kidney cell culture (Brotherston and Boyce, 1970), the methanol precipitated protein was resuspended in PBS supplemented with 10 per cent

inactivated sheep serum and the resulting concentrates were emulsified with oil adjuvant containing phenol at a concentration of 0.3 per cent (v/v). The concentration of phenol in this vaccine was thus less than in the presently available vaccine. Unfortunately however the effect of long term storage on the antigenic stability of either concentrate or emulsified vaccine was not determined.

Despite the antigenic instability of the vaccine emulsion, commercial production of louping-ill vaccine using the technique of ultrafiltration to concentrate viral antigen must be regarded as a very attractive alternative to the present method of production. Further studies outwith this project are however required to improve the storage characteristics of the vaccine emulsion and it is suggested that these be designed to assess the stability of phenol-free vaccine emulsions and to evaluate the use of alternative adjuvants, preservative agents and storage conditions.

Table 9.1 Established and experimental arthropod-transmitted Togavirus vaccines

Vaccine	Method of virus propagation	Reference
Formalin inactivated Eastern equine encephalitis	Primary chick embryo cell culture	Maire, McKinney and Cole, 1970
Formalin inactivated Western equine encephalitis	Primary chick embryo cell culture	Bartelloni, McKinney, Calia, Ramsburg and Cole, 1971
Formalin inactivated Venezuelan equine encephalitis (derived from an attenuated vaccine strain)	Primary chick embryo cell culture	Edelman, Ascher, Oster, Ramsburg, Cole and Eddy, 1979
Formalin inactivated Chikungunya	Green monkey kidney cell culture	Harrison, Eckels, Bartelloni and Hampton, 1971
	Concentrated chick embryo suspension culture	White, Berman and Lowenthal, 1972
Formalin inactivated Japanese B* encephalitis	Mouse brain	Kamitsu, Hashimoto, Urasawa, Kataurada and Kimura, 1970
Formalin inactivated Japanese B encephalitis (derived from an attenuated strain)	Hamster kidney cell culture	Darwish and Hamman, 1966b
Live attenuated Japanese B* encephalitis	Monkey kidney cell culture	Ueba, Kimura, Nakajima, Kurimura, Kitaura, 1978

*Established vaccines

Table 9.1 cont'd. Established and experimental arthropod-transmitted Togavirus vaccines

Vaccine	Method of virus propagation	Reference
Live Yellow Fever*	Chick embryo, mouse brain	British Pharmaceutical Codex 1979
Attenuated Dengue Type 2	Green monkey kidney cell culture	Harrison, Eckels, Sagartz and Russell, 1977
Attenuated Turkey meningo-encephalitis*	Embryonated chick egg	Komarov and Kalmar, 1960
Formalin inactivated louping-ill*	BHK-21 cell culture	British Pharmacopoeia (Veterinary) 1977
Formalin inactivated tick-borne encephalitis*	Chick embryo cell culture	Kunz, Hoffmann and Stary, 1976
	Mouse brain, chick embryo cell culture	Levkovich, 1962
Attenuated tick-borne encephalitis (derived from Langat E5 virus)	Embryonated chick egg	Price, Thind, Teasdall and O'Leary 1970 Mayer, Pogady, Stárek and Hrbka, 1975

*Established vaccines

REFERENCES

Adam, K.M.G., Beasley, S.J. and Blewett, D.A. 1977. The occurrence of antibody to Babesia and to the virus of louping-ill in deer in Scotland. Res. Vet. Sci., 23, 133-138.

Adams, A. 1973. Concentration of Epstein-Barr virus from cell culture fluids with polyethylene glycol. J. Gen. Virol., 20, 391-394.

Alexander, R.A. and Neitz, W.O. 1933. The transmission of louping-ill of sheep by ticks (Rhipicephalus appendiculatus). Vet. J., 89, 320-323.

Alston, J.M. and Gibson, H.J. 1931 Note on experimental transmission of 'louping-ill' to mice. Br. J. Exp. Pathol., 12, 82-88.

Andrewes, C.H. and Horstmann, D.W. 1949. The susceptibility of viruses to ethyl ether. J. Gen. Micro., 3, 290-297.

Andrewes, C., Pereira, H.G. and Wildy, P. 1978. In: Viruses of Vertebrates, 4th Edition. Ballière Tindall, London. 91-94.

Appleyard, G., Oram, J.D. and Stanley, J.L. 1970. Dissociation of Semliki Forest Virus into biologically active components. J. Gen. Virol., 9, 179-189.

Arthur, D.R. 1963. British Ticks. Butterworths, London.

Bannatyne, C.C., Wilson, R.L., Reid, H.W., Buxton, D. and Pow, I. 1980. Louping-ill virus infection of pigs. Vet. Rec., 106, 13.

Bartelloni, P.J., McKinney, R.W., Calia, F.M., Ramsburg, H.H. and Cole, F.E. 1971. Inactivated Western Equine Encephalomyelitis vaccine propagated in chick embryo cell culture. Clinical and serological evaluation in man. Am. J. Trop. Med. Hyg., 20, 146-149.

Beasley, S.J., Campbell, J.A. and Reid, H.W. 1978. Threshold problems in infection of Ixodes ricinus with the virus of louping-ill. in: Wilde, J.K.H. Proceedings of the International Conference on tick-borne diseases and their vectors. Edinburgh University Press, 497-500.

Bijlenga, G. and Van Den Bogaard, A.E.J.M. 1973. In vivo enhancement of rabies infection by diethylaminoethyl-dextran. Archiv für die gesamte Virusforschung., 42, 96-101.

Bose, H.R. and Sagik, B.P. 1970. Immunological activity associated with the nucleocapsid and envelope components of an arbovirus. J. Virol., 5, 410-412.

Brambell, F.W.R. 1958. The passive immunity of the young mammal. Biological Reviews, 33, 488-531.

Brewis, E.G., Neubauer, C. and Hurst, E.W. 1949. Another case of louping-ill in man; isolation of virus. Lancet, 1, 689-691.

Bronson, D.L., Elliott, A.Y. and Ritzi, D. 1975. Concentration of Rous sarcoma virus from tissue culture fluids with polyethylene glycol. App. Microbiol. 30, 464-471.

Brotherston, J.G., Bannatyne, C.C., Mathieson, A.O. and Nicholson, T.B. 1971. Field trials of an inactivated oil-adjuvant vaccine against louping-ill (Arbovirus group B). J. Hyg., (Camb), 69, 479-489.

Brotherston, J.G. and Boyce, J.B. 1970. Development of a non-infective protective antigen against louping-ill (Arbovirus group B). Laboratory experiments. J. Comp. Path., 80, 377-388.

Brown, F., Smale, C.J. and Horzinek, M.C. 1974. Lipid and protein organisation in vesicular stomatitis and Sindbis viruses. J. Gen. Virol., 22, 455-458.

Brownlee, A. and Wilson, D.R. 1932. Studies in the histopathology of louping-ill. J. Comp. Path. Ther., 45, 67-92.

Burfoot, C., Young, P.A. and Finter, N.B. 1977. The thermal stability of a stabilized 17D yellow fever virus vaccine. J. Biol. Stand., 5, 173-179.

Burge, B.W. and Huang, A.S. 1970. Comparison of membrane protein glycopeptides of Sindbis virus and vesicular stomatitis virus. J. Virol., 6, 176-182.

Burge, B.W. and Strauss, J.H. 1970. Glycopeptides of the membrane glycoprotein of Sindbis virus. J. Mol. Biol., 47, 449-466.

Burnet, F.M. 1936. Observations on the effect of louping-ill virus on the developing egg. Br. J. Exp. Pathol., 17, 294-301.

Burnet, F.M. and Lush, D. 1940 The action of certain surface active agents on viruses. Austr. J. Exptl. Biol. Med. Sci., 18, 141-150.

Butler, M. and Leach, R.H. 1964. A mycoplasma which induces acidity and cytopathic effect in tissue culture. J. Gen. Microbiol. 34, 285-294.

Buxton, D., Reid, H.W. and Pow, I. 1979. Immunosuppression in toxoplasmosis: studies in mice with a clostridial vaccine and louping-ill virus vaccine. J. Comp. Path., 89, 375-379.

Charney, J., Fisher, W.P. and Machlowitz, R.A. 1957. Preparation and inactivation of purified poliovirus: Comparison of vaccines derived from Mahoney and Parker poliovirus. Proc. Soc. Exp. Bio. Med., 96, 601-605.

Clarke, D.H. and Casals, J. 1958. Techniques for haemagglutination and haemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg., 7, 561-573.

Cleaver, J.E. 1974. Plaque formation with Simian virus 40: Enhancement by dimethylsulfoxide. J. Virol., 14, 1607-1610.

Cleishbottam, J. 1816. The black dwarf. In: Tales of my landlord. Vol. 1. William Blackwood, Edinburgh.

Cole, F.E., May, S.W. and Robinson, D.M. 1973. Formalin-inactivated Venezuelan equine encephalomyelitis (Trinidad strain) vaccine produced in rolling-bottle cultures of chicken embryo cells. App. Micro., 25, 262-265.

Cox, H.R., Van der Scheer, J., Aiston, S. and Bohnel, E. 1947. The purification and concentration of influenza virus by means of alcohol precipitation. J. Immunol., 56, 149-166.

Crawford, J.G., Dayhuff, T.R. and White, E.A. 1968. Hog cholera: Safety and protection studies with phytodynamically inactivated hog cholera virus. Am. J. Vet. Res. 29, 1749-1759.

Czarkowska-Gladney, J. and Hurst, E.W. 1931. Some data concerning the infectivity, survival and powers of diffusion of the virus of 'louping-ill. Br. J. Exp. Pathol., 12 426-430.

Danes, L. and Benda, R. 1960. Study of the possibility of preparing a vaccine against tick-borne encephalitis using tissue culture methods. 11. The inactivation by formaldehyde of the TBE virus in liquids prepared from tissue culture. Immunogenic properties: Acta. Virol., 4, 82-93.

Darwish, M.D. and Hammon, W.M. 1966a. Studies on Japanese B encephalitis virus vaccines from tissue culture. VI Development of a hamster kidney tissue culture inactivated vaccine for man (2) The characteristics of inactivation of an attenuated strain of OCT-541. J. Immunol., 96, 806-813.

Darwish, M.D. and Hammon, W.M. 1966b. Studies on Japanese B encephalitis virus vaccines from tissue culture. VI Development of a hamster kidney tissue culture inactivated vaccine for man (3) Potency testing of an attenuated strain of OCT-541. J. Immunol., 96, 878-884.

Darwish, M.D. and Hammon, W.M. 1966c. Japanese B encephalitis virus vaccines from tissue culture. VII Formalin inactivated Nakayama strain vaccine. Proc. Soc. Exp. Biol., 122. 813-816.

Davey, M.W., Dennett, D.P. and Dalgarno, L. 1973. The growth of two togaviruses in cultured mosquito and vertebrate cells. J. Gen. Virol., 20, 225-232.

David, A.E. 1971. Lipid composition of Sindbis virus. Virology, 46, 711-720.

Davison, G., Neubauer, C. and Hurst, E.W. 1948. Meningo-encephalitis in man due to louping-ill virus. Lancet, 2, 453-457.

Della-Porta, A.J. and Westaway, E.G. 1972. Rapid preparation of haemagglutinins of togaviruses from infected cell culture fluids. App. Microbiol. 23, 158-160.

Doherty, P.C. 1969a. Effect of age on louping-ill encephalitis in the hamster. J. Comp. Path. 79, 413-420.

Doherty, P.C. 1969b. Louping-ill encephalomyelitis in C57 black mice. J. Comp. Path. 79, 571-576.

Doherty, P.C, and Reid, H.W. 1971a. Experimental louping-ill in sheep and lambs. II Neuropathology. J. Comp. Path. 81. 331-337.

Doherty, P.C. and Reid, H.W. 1971b. Louping-ill encephalomyelitis in the sheep. II Distribution of virus and lesions in the nervous tissue. J. Comp. Path. 81, 531-536.

Dow, C. and McFerran, J.B. 1964. The neuropathology of experimental louping-ill in pigs. Res. Vet. Sci., 5, 32-38.

Dulbecco, R. and Vogt, M. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med., 99, 167-199.

Duncan, A. 1807. A treatise on the diseases of sheep drawn up from original communications presented to the Highland Society of Scotland. Trans. Highl. Agric. Soc. Scot., 3, 339-380.

Dunn, A.M. 1952. Louping-ill. A study of the disease in cattle. Ph.D. Thesis, University of Edinburgh.

Dunn, A.M. 1960. Louping-ill - the red deer (cervus elaphus) as an alternative host of the virus in Scotland. Br. Vet. J., 116, 284-287.

Dunnett, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. J. Amer. Statist. Ass., 50, 1096-1121.

Edelman, R., Ascher, M.S., Oster, C.N., Ramsburg, H.H., Cole, F.E. and Eddy, G.A. 1979. Evaluation in humans of a new inactivated vaccine for Venezuelan equine encephalitis virus. J. Infect. Dis., 140, 708-715.

Edward, D.G. 1947a. Culture of louping-ill virus in the embryonated egg. Br. J. Exp. Pathol., 28, 237-247.

Edward, D.G. 1947b. Methods for investigating immunization against louping-ill. Br. J. Exp. Pathol., 28, 368-376.

Edward, D.G. 1948a. Immunization against louping-ill. Vaccines prepared from chick embryos. Br. J. Exp. Pathol., 29, 367-372.

Edward, D.G. 1948b. Immunization against louping-ill. Immunization of man. Br. J. Exp. Pathol., 29, 372-378.

Elford, W.J., Chu, C.M., Dawson, I.M., Dudgeon, J.A., Fulton, F. and Smiles, J. 1948. Physical properties of the viruses of Newcastle disease, fowl plague and mumps. Br. J. Exp. Pathol., 29, 590-599.

Elford, W.J. and Galloway, I.A. 1933. Size of virus of louping-ill of sheep by method of ultrafiltration analysis. J. Pathol. Bact., 37, 381-392.

Findlay, G.M. and Elton, C. 1933. The transmission of louping-ill in field voles. J. Comp. Path. Ther., 46, 126-128.

Fletcher, J.M. and Galloway, I.A. 1937. Louping-ill in the horse. Vet. Rec., 49, 17-18.

Friedmann, T. and Haas, M. 1970. Rapid concentration and purification of Polyoma virus and SV40 with polyethylene glycol. Virology, 42, 248-250.

Frimmhagen, L.H. and Knight, C.A. 1959. Column purification of influenza virus. Virology, 8, 198-208.

Gall, W. 1973. Studies on the immunizing potency of tick-borne encephalitis vaccines. Acta. Microbiologica Polonica, 5. 279-285.

Garoff, H. 1974. Cross linking of the spike glycoproteins in Semliki Forest virus with dimethylsuberimidate. Virology, 62, 385-392.

Garoff, H. and Simons, K. 1974. Location of the spike glycoproteins in the Semliki Forest virus membrane. Proc. Nat. Acad. Sci. USA, 71, 3988-3992.

Garoff, H., Simons, K. and Renkonen, O. 1974. Isolation and characterization of the membrane proteins of Semliki Forest virus. Virology, 61, 493-504.

Garwes, D.J. and Pocock, D.H. 1975. Effect of precipitation with methanol on antigenic potency of TGE virus. Vet. Rec. 97, 34.

Gordon, W.S. 1934. The control of certain diseases of sheep. Vet. Rec. 14, 1-12.

Gordon, W.S. 1946. Advances in veterinary research. Vet. Rec., 58, 516-525.

Gordon, W.S., Brownlee, A., Wilson, D.R. and MacLeod, J. 1932. Studies in louping-ill (an encephalomyelitis of sheep). A. A note on the infectivity of blood. B. A field experiment (1931) with a preliminary note on the nature of tick-borne fever. J. Comp. Path. Ther. 45, 106-140.

Gordon, W.S., Brownlee, A., Wilson, D.R. and MacLeod, J. 1962. The epizootiology of louping-ill and tick-borne fever with observations on the control of these sheep diseases. Symp. Zool. Soc. Lond., No. 6, 1-27.

Greig, J.R., Brownlee, A., Wilson, D.R. and Gordon, W.S. 1931. Nature of louping-ill. Vet. Rec., 11, 325-333.

Grešíková, M., Albrecht, P. and Ernek, E. 1961. Comparison of an attenuated and virulent louping-ill strain. Nature, 190, 508-510.

Guskey, L.E. and Wolff, D.A. 1972. Concentration and purification of Poliovirus by ultrafiltration and isopycnic centrifugation. App. Microbiol., 24, 13-17.

Harris, R.J.C. 1964. Techniques in experimental virology, p. 157, Academic Press, London,

Harrison, S.C., David, A., Jumblatt, J. and Darnell, J.E. 1971. Lipid and protein organization in Sindbis virus. J. Mol. Biol., 60, 523-528.

Harrison, V.R., Binn, L.N. and Randall, R. 1967. Comparative immunogenicities of Chikungunya vaccines prepared in avian and mammalian tissues. Amer. J. Trop. Med. Hyg., 16, 786-791.

Harrison, V.R., Eckels, K.H., Bartelloni, P.J. and Hampton, C. 1971. Production and evaluation of a formalin-killed Chikungunya vaccine. J. Immunol., 107, 643-647.

Harrison, V.R., Eckels, K.H. Sagartz, J.W. and Russell, P.K. 1977. Virulence and immunogenicity of a temperature sensitive Dengue-2 virus in lower primates. Infect. and Immun., 18, 151-156.

Hebert, T.T. 1963. Precipitation of plant viruses by polyethylene glycol. Phytopathology, 53, 361-366.

Heinz, F. and Kunz, Ch. 1977a. Concentration and purification of tick-borne encephalitis virus grown in suspensions of chick embryo cells. Acta. Virol., 21, 301-307.

Heinz, F. and Kunz, Ch. 1977b. Characterization of tick-borne encephalitis virus and immunogenicity of its surface components in mice. Acta Virol., 21, 308-316.

Heydrick, F.P., Comer, J.F. and Wachter, R.J. 1971. Phospholipid composition of Venezuelan equine encephalitis virus. J. Virol., 7, 642-645.

Horzinek, M.C. 1969. A simple method for concentration of arboviruses propagated in tissue culture. Am. J. Trop. Med. Hyg., 18, 588-591.

Horzinek, M.C. 1973. Comparative aspects of togaviruses. J. Gen. Virol., 20, 87-103.

Hurst, E.W. 1931. The transmission of 'louping-ill' to the mouse and the monkey: histology of the experimental disease. J. Comp. Path. Ther., 44, 231-245.

Igarashi, A., Fukuoka, T., Sasao, F., Surimarut, S. and Fukai, K. 1973. Purification of Japanese encephalitis virus grown in BHK-21 and Singh's Aedes albopictus cells by polyethylene glycol precipitation. Biken Journal, 16, 67-73.

Inglot, A.D., Chudzio, T. and Albin, M. 1973. Analysis of Sindbis virus and its soluble antigens in preparations concentrated by precipitation with polyethylene glycol or ammonium sulphate. Acta. Virol., 17, 416-425.

Jensen, M.T., Kemeny, L.J. and Stone, S.S. 1979. Methanol precipitation of transmissible gastroenteritis virus. Am. J. Vet. Res., 40, 1798-1799.

Kamitsu, M., Hashimoto, N., Urasawa, S., Kataurada, M. and Kimura, H. 1970. A field trial with an improved Japanese encephalitis vaccine in a non-endemic area of the disease. Biken Journal, 13, 313-328.

Kanarek, A.D. and Tribe, G.W. 1967. Concentration of certain myxoviruses with polyethylene glycol. Nature, 214, 927-928.

Karabatsos, N. and Buckley, S.M. 1967. Susceptibility of the baby hamster kidney line (BHK-21) to infection with arboviruses. Am. J. Trop. Med. Hyg., 16, 99-105.

Kennedy, S.I.T. 1974. The effect of enzymes on structural and biological properties of Semliki Forest virus. J. Gen. Virol., 23, 129-143.

Kitano, T., Suzuki, K. and Yamaguchi, T. 1974. Morphological, chemical and biological characterization of Japanese encephalitis virus virion and its haemagglutinin. J. Virol., 14, 631-639.

Klein, F., Mahlandt, B.G., Bonner, H.B. and Lincoln, R.E. 1971. Ultrafiltration as a method for concentrating Rift Valley fever virus grown in tissue culture. Appl. Microbiol. 21, 758-760.

Komarov, A. and Kalmar, E. 1960. A hitherto undescribed disease - Turkey Meningo-encephalitis. Vet. Rec. 72, 257-261.

Koprowski, H. and Cox, H.R. 1946. Studies on chick embryo vaccines against Japanese B encephalitis. J. Immunol., 54, 357-370.

Kunz, Ch, Hofmann, H. and Stary, D.H. 1976. Field studies with a vaccine against tick-borne encephalitis. Zbl. Bakt. Hyg. I. Abt. Orig., 234, 141-144.

Laidlaw, R. 1872. On the natural history of the sheep tick. Transactions of the Highland and Agricultural Society of Scotland, Fourth Series, Vol. IV, 205-208.

Laidlaw, P.P. and Dunkin, G.W. 1928. Studies in dog distemper. IV The immunization of ferrets against dog distemper. J. Comp. Path., 41, 1-17.

Laine, R., Kettunen, M., Gahmberg, C.G., Kääriäinen, L. and Renkonen, O. 1972. Fatty chains of different lipid classes of Semliki Forest virus and host cell membranes. J. Virol., 10, 433-438.

Lawson, J.H., Manderson, W.G. and Hurst, E.W. 1949. Louping-ill meningo-encephalitis; further case and serological survey. Lancet, 2, 696-699.

Leberman, R. 1966. The isolation of plant viruses by means of 'simple' coacervates. Virology, 30, 341-347.

Lépine, P. 1931. Etude des proprietes du virus de la tremblante du mouton. C.R. Soc. Biol. Paris, 108, 476-478.

Levkovich, E.N. 1962. Experimental and epidemiological basis of the specific prophylaxis of tick-borne encephalitis In: Biology of viruses of the tick-borne encephalitis complex. Czechoslovak Academy of Sciences Praha, 317-329.

Levkovich, E.N. and Karpovich, L.G. 1962. Study on biological properties of viruses of the tick-borne encephalitis complex in tissue cultures. In: Biology of viruses of the tick-borne encephalitis complex. Czechoslovak Academy of Sciences Praha, 161-165.

Libíková, H. and Albrecht, P. 1962. Study of the reproduction of tick-borne encephalitis virus in HeLa cells by biological, immunohistological and morphological methods. In: Biology of viruses of the tick-borne encephalitis complex, Czechoslovak Academy of Sciences Praha, 171-175.

Likar, M. and Dane, D.S. 1958. An illness resembling acute poliomyelitis by the RSSE/louping-ill group in N. Ireland. Lancet, 1, 456-458.

MacKenzie, C.P., Smith, S.T. and Muir, R.W. 1973. Louping-ill in a working collie. Vet. Rec., 92, 354-356.

MacLeod, J. and Gordon, W.S. 1932. Studies in louping-ill (encephalomyelitis of sheep), transmission by sheep tick Ixodes ricinus L. J. Comp. Path. Ther., 45, 240-256.

MacPherson, I. and Stoker, M. 1962. Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. Virology, 16, 147-151.

Maire, L.F., McKinney, R.W. and Cole, F.E. 1970. An inactivated Eastern equine encephalomyelitis vaccine propagated in chick embryo cell culture. 1. Production and testing. Am. J. Trop. Med. Hyg., 19, 119-122.

Malewicz, B. and Jenkin, H.M. 1979. Cultivation of Dengue virus type 2 in baby hamster kidney cells in serum-free medium. Am. J. Trop. Med. Hyg., 28, 918-920.

Maurer, F.D., Walker, R.V.L., Shope, R.E., Griffiths, H.J. and Jenkins, D.L. 1946. Attempts to prepare an effective Rinderpest vaccine from inactivated egg-cultivated virus. Am. J. Vet. Res., 7, 164-169.

Mayer, V., Blaškovič, D, Ernek, E. and Libíková, H. 1969. The serological response and long lasting resistance against infection with louping-ill virus in sheep immunized with a highly attenuated tick-borne encephalitis virus. J. Hyg. (Camb.), 67, 731-738.

Mayer, V., Pogady, J., Stárek, M. and Hrbka, J. 1975. A live vaccine against tick-borne encephalitis: Integrated studies, III Response of man to a single dose of the E5 '14' clone (Langat virus). Acta. Virol., 19, 229-236.

Mayer, V., Zemla, J. and Albrecht, A. 1962. Growth characteristics of a tick-borne encephalitis virus in suspended HeLa cell cultures. In: Biology of viruses of the tick-borne encephalitis complex. Czechoslovak Academy of Sciences Praha, 180-182.

McCutchan, J.H. and Pagano, J.S. 1968 Enhancement of the infectivity of Simian Virus 40 deoxyribonucleic acid with diethylamino-ethyl dextran. J. Nat. Cancer Inst., 41, 351-356.

McSharry, J. and Benzinger, R. 1970. Concentration and purification of vesicular stomatitis virus by polyethylene glycol 'precipitation'. Virology, 40, 745-746.

M'Fadyean, J. 1894. Louping-ill in sheep. J. Comp. Path. Ther., 7, 207-219.

M'Fadyean, J. 1900. The etiology of louping-ill. J. Comp. Path. Ther., 13, 145-154.

Midulla, M., Wallis, C. and Melnick, J.L. 1965. Enterovirus immunizing antigens in the form of cation-stabilized and concentrated virus preparations. J. Immunol., 95, 9-12.

Miller, H.K. and Schlesinger, R.W. 1955. Differentiation and purification of influenza viruses by adsorption on aluminium phosphate. J. Immunol., 75, 155-160.

Morrow, A.W., Whittle, C.J. and Eales, W.A. 1974. A comparison of methods for the concentration of foot and mouth disease virus for vaccine preparation. Bull. Off. Int. Epiz., 81, 1155-1167.

Mussgay, M., Weiland, E., Weiland, F. and Härtner, D. 1976). Some properties of the surface of formalin-inactivated Semliki Forest virus after treatment with Tween 80 - Tri (n-Butyl) phosphate or saponin. Zbl. Vet. Med. B., 23, 447-457.

Norrby, E.C.J. and Albertsson, P.A. 1960. Concentration of poliovirus by an aqueous polymer two-phase system. Nature, 188, 1047-1048.

Oker-Blom, N. 1956. Propagation of louping-ill virus in malignant human epithelial cells, strain HeLa. Ann. Med. Exptl. Biol. Penn., 34, 199-205.

O'Reilly, K.J., Smith, C.E.G., McMahon, D.A., Wilson, A.L. and Robertson, J.M. 1965. Infection of sheep and monkeys with Langat virus, cross protection against other viruses of the Russian spring-summer complex. J. Hyg. (Camb.), 63, 213-221.

Pattyn, S.R. and Vleesschauwer, L. De. 1969. Plaque production by Group A arboviruses. Enhancing effect of DEAE-Dextran on plaque numbers in chick embryo cells. Acta. Virol., 13, 26-33.

Pedersen, C.E. and Eddy, G.A. 1974. Separation, isolation and immunological studies of the structural proteins of Venezuelan equine encephalomyelitis virus. J. Virol., 14, 740-744.

Pedersen, C.E., Slocum, D.R. and Eddy, G.A. 1973. Immunological studies on the envelope component of Venezuelan equine encephalomyelitis virus. Inf. Imm., 8, 901-906.

Philipson, L., Albertsson, P.A. and Frick, G. 1960. The purification and concentration of viruses by aqueous polymer phase systems. Virology, 11, 553-571.

Pollard, M., Connolly, J. and Fromm, S. 1949. The precipitating effect of methanol on viruses. Proc. Soc. Exp. Biol. Med., 71, 290-293.

Pool, W.A., Brownlee, A. and Wilson, R.D. 1930. The etiology of 'louping-ill'. J. Comp. Path. Ther., 43, 253-290.

Poste, G. 1970. Virus induced polykaryocytosis and the mechanism of cell fusion. Adv. Virus. Res., 16, 303-356.

Poste, G. 1972. Mechanism of virus induced cell fusion. Int. Rev. Cytol., 33, 157-252.

Poste, G. and Allison, A.C. 1971. Membrane fusion reaction, a theory. J. Theor. Biol., 32, 165-184.

Price, W.H., Thind, I.S., Teasdall, R.D. and O'Leary, W. 1970. Vaccination of human volunteers against Russian spring-summer (RSS) virus complex with attenuated Langat E5 virus. Bull. Wld. Hlth. Org., 42, 89-94.

Quigley, J.P., Rifkin, D.B. and Reich, E. 1971. Phospholipid composition of Rous sarcoma virus, host cell membranes and other enveloped RNA viruses. Virology, 46, 106-116.

Reed, S.E., 1971. The interaction of mycoplasmas and influenza viruses in tracheal organ cultures. J. Infect. Dis., 124, 18-25.

Reid, H.W. 1975a. A study of the pathogenesis of louping-ill. Ph.D. thesis, Edinburgh University.

Reid, H.W. 1975b. Experimental infection of red grouse with louping-ill virus (flavivirus group). I. The viraemia and antibody response. J. Comp. Path. 85, 223-229.

Reid, H.W. 1978. The epidemiology of louping-ill. In: Wilde, J.K.H. Proceedings of the International Conference on tick-borne diseases and their vectors. Edinburgh University Press, 501-507.

Reid, H.W., Barlow, R.M., Boyce, J.B. and Inglis, D.M. 1976. Isolation of louping-ill virus from a roe deer (Capreolus capreolus). Vet. Rec., 98, 116.

Reid, H.W. and Boyce, J.B. 1974. Louping-ill virus in red grouse in Scotland. Vet. Rec., 95, 150.

Reid, H.W. and Boyce, J.B. 1976. The effect of colostrum-derived antibody on louping-ill virus infection in lambs. J. Hyg. (Camb.), 77, 349-354.

Reid, H.W. and Doherty, P.C. 1971a. Experimental louping-ill in sheep and lambs. 1. Viraemia and the antibody response. J. Comp. Path., 81, 291-298.

Reid, H.W. and Doherty, P.C. 1971b. Louping-ill encephalomyelitis in the sheep. 1. The relationship of viraemia and the antibody response to susceptibility. J. Comp. Path., 81, 521-529.

Reid, H.W., Doherty, P.C. and Dawson, A. McL. 1971. Louping-ill encephalomyelitis in the sheep. III Immunoblogulins in cerebrospinal fluid. J. Comp. Path., 81, 537-543.

Reid, H.W., Moss, R., Pow, I. and Buxton, D. 1980. The response of three grouse species (Tetrao Urugallus, Lagopus mutus, Lagopus lagopus) to louping-ill virus. J. Comp. Path., 90, 257-263.

Rhim, J.S., Williams, L.B., Huebner, R.J. and Turner, H.C. 1969. Concentration by Diaflo ultrafiltration of murine leukemia and sarcoma viruses grown in tissue cultures. Cancer. Res., 29, 154-156.

Rivers, T.M. and Schwentker, F.F. 1933. Louping-ill in man. Proc. Soc. Exp. Biol. Med., 30, 1302-1303.

Rivers, T.M. and Schwentker, F.F. 1934. Louping-ill in man. J. Exp. Med., 59, 669-685.

Rivers, T.M. and Ward, S.M. 1933. Cultivation of louping-ill virus. Proc. Soc. Exp. Biol. Med., 30, 1300-1301.

Robinson, D.M., Berman, S., Lowenthal, J.P. and Hetrick, F.M. 1966. Western equine encephalomyelitis vaccine produced in chick embryo cell cultures. Appl. Microbiol., 14, 1011-1014.

Ross, C.A.C. 1961. Louping-ill in the west of Scotland. Lancet, 2, 527-528.

Rossi, C.R. 1971. Studies of Laryngotracheitis virus in avian tissue culture. IV Effect of diethylaminoethyl-Dextran on adsorption and elution. Archiv. für die gesamte virus-forschung., 35, 223-231.

Rossi, C.R. and Kiesel, G.K. 1978. Bovine respiratory syncytial virus infection of bovine embryonic lung cultures. Enhancement of infectivity with DEAE and virus infected cells. Arch. Virol., 56, 227-236.

Rouse, H.C., Bonifas, V.H. and Schlesinger, R.W. 1963. Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. Virology, 20, 357-365.

Rubin, H., Franklin, R.M. and Baluda, M. 1957. Infection and growth of Newcastle disease virus (NDV) in cultures of chick embryo lung epithelium. Virology, 3, 587-600.

Sabin, A.B. 1932. Experiments on the purification and concentration of the virus of poliomyelitis. J. Exp. Med., 56, 307-317.

Salk, J.E. 1941. Partial purification of the virus of epidemic influenza by adsorption on calcium phosphate. Proc. Soc. Exp. Biol. Med., 46, 709-712.

Salk, J.E. and Gori, J.B. 1960. A review of theoretical, experimental and practical considerations in the use of formaldehyde for the inactivation of poliovirus. Ann. N.Y. Acad. Sci., 83, 609-637.

Schaffer, F.L. 1960. Interaction of poliovirus with formaldehyde. Ann. N.Y. Acad. Sci., 83, 564-577.

Schaeffer, M. and Brebner, W.B. 1933. Purification of poliomyelitis virus. Arch. Pathol., 15, 221-226.

Schlesinger, M.J., Schlesinger, S. and Burge, B.W. 1972. Identification of a second glycoprotein in Sindbis virus. Virology, 47. 539-541.

Schwentker, F.F., Rivers, T.M. and Finkelstein, M.H. 1933. Observations on the immunological relation of poliomyelitis to louping-ill. J. Exp. Med., 57, 955-965.

Schwerdt, C.E. and Schaffer, F.L. 1956. Purification of poliomyelitis viruses propagated in tissue culture. Virology, 2, 665-678.

Seamer, J. and Zlotnik, I. 1970. Louping-ill and Semliki Forest virus infections in the short tailed vole Microtus agrestis (L). Br. J. Exp. Pathol., 51, 385-393.

Shigeko, N. 1968. Infection of respiratory syncytial virus with polyions: Enhancement of infectivity with diethylaminoethyl dextran. Proc. Soc. Exp. Biol. Med., 128, 163-166.

Singer, S.H., Barile, M.F. and Kirschstein, R.L. 1969. Enhanced virus yields and decreased interferon production in mycoplasma-infected hamster cells. Proc. Soc. Exp. Biol. Med. 131, 1129-1134.

Singer, S.H., Fitzgerald, E.A., Barile, M.F. and Kirschstein, R.L. 1970. Effect of mycoplasmas on vaccine virus growth. Requirement for arginine. Proc. Soc. Exp. Biol. Med., 133, 1439-1442.

Singer, S.H., Kirschstein, R.L. and Barile, M.F. 1969. Increased yields of stomatitis virus from hamster cells injected with mycoplasma. Nature, 222, 1087-1088.

Smith, C.E.G. 1969. Arbovirus vaccines, Br. Med. Bull., 25, 142-147.

Smith, C.E.G., McMahon, D.A., O'Reilly, J.K., Wilson, A.L. and Robertson, J.M. 1964. The epidemiology of louping-ill in Ayrshire: the first year of studies in sheep. J. Hyg. (Camb.), 62, 53-68.

Smith, C.E.G., Varma, M.G.R. and McMahon, D. 1964. Isolation of louping-ill virus from small mammals in Ayrshire, Scotland. Nature, 203, 992-993.

Smith, W. 1939. The action of bile salts on viruses. J. Path. Bact., 48, 557-571.

Smorodinstev, A. and Ilyenko, V. 1962. Results of laboratory and epidemiological study of vaccination against tick-borne encephalitis. In: Biology of viruses of the tick-borne encephalitis complex. Czechoslovak Academy of Sciences Praha, 332-343.

Stow, N.D. and Wilkie, N.M. 1976. An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J. Gen. Virol., 33, 447-458.

Strauss, J.H., Burge, B.W. and Darnell, J.E. 1970. Carbohydrate content of the membrane protein of Sindbis virus. J. Mol. Biol., 47, 437-448.

Swanepoel, R, 1967-1968. Quantitative studies on the virus of louping-ill in sheep and tick. Ph.D. Thesis, University of Edinburgh.

Thormar, H., Wisniewski, H.M. and Lin, F.H. 1979. Sera and cerebrospinal fluids from normal uninfected sheep contain a visna virus inhibiting factor. Nature, 279, 245-246

Timm, E.A., MacLean, I.W., Kupsky, C.H. and Hook, A.E. 1956. The nature of the formalin inactivation of poliomyelitis virus. J. Immun., 77, 444-452.

Timoney, P.J. 1980. Susceptibility of the horse to experimental inoculation with louping-ill virus. J. Comp. Path. 90, 73-86.

Traub, E. 1938. Immunization of guinea pigs against lymphocytic choriomeningitis with formalinized tissue vaccines. J. Exp. Med., 68, 95-110.

Ueba, N., Kimura, T., Nakajima, S., Kurimura, T. and Kitaura, T. 1978. Field experiments on live attenuated Japanese encephalitis virus vaccine for swine. Biken Journal, 21, 95-103.

Utermann, G. and Simons, K. 1974. Studies on the amphipathic nature of the membrane proteins in Semliki Forest virus. J. Mol. Biol., 85, 569-587.

Vaheri, A., Sedwick, W.D. and Plotkin, S.A. 1967. Growth of rubella virus in BHK-21 cells. II Enhancing effect of DEAE dextran, semicarbazide and low doses of metabolic inhibitors. Proc. Soc. Exp. Biol. Med., 125, 1092-1098.

Van Wezel, A.L., 1974. In "Dialog' Oosterhout. Amicon B.V. Technical Publication No. 9.

Volkert, M. and Horsfall, F.L. 1947. Studies on a lung tissue component which combines with pneumonia virus of mice (PVM). J. Exp. Med., 86, 393-407.

Von Zeipel, G. and Svedmyr, A. 1958. Growth of viruses of the Russian spring Summer - louping-ill group in tissue culture, Archiv. für die gesamte virusforschung., 8, 370-384.

Wagner, G.G., Card, J.L. and Cowan, K.M. 1970. Immunochemical studies of foot and mouth disease. VII Characterization of foot and mouth disease virus concentrated by polyethylene glycol precipitation. Archiv. für die gesamte virusforschung., 30, 343-352.

Webb, H.E. Connolly, J.H., Kane, F.F., O'Reilly, K.J. and Simpson, D.I. 1968. Laboratory infections with louping-ill with associated encephalitis. Lancet, 2, 255-260.

Webster, L.T. and Fite, G.L. 1933. Infection in mice following instillation of louping-ill virus. Proc. Soc. Exp. Biol. Med., 30, 656-657.

Weiss, S.A. 1980. Hollow fibre ultrafiltration technology used for concentration of baboon endogenous virus in large scale production. Biotech. Bioeng., 22, 19-31.

Wells, P.W. and Reid, H.W. 1978. Antibody responses to vaccination against louping-ill virus in newborn lambs. J. Comp. Path., 88, 425-431.

White, A., Berman, S. and Lowenthal, J.P. 1971. Inactivated Eastern equine encephalomyelitis vaccines prepared in monolayer and concentrated suspension chick embryo culture. App. Microbiol., 22, 909-913.

White, A., Berman, S. and Lowenthal, J.P. 1972. Comparative immunogenicities of Chikungunya vaccines propagated in monkey kidney monolayers and chick embryo suspension cultures. App. Micro., 23, 951-952.

Williams, H.E. 1958. Growth and titration of louping-ill virus in monolayer tissue culture of pig kidney. Nature, 181, 497-498.

Williams, H. and Thorburn, H. 1961. The serological response of sheep to infection with louping-ill virus. J. Hyg. (Camb.), 59, 437-447.

Williams, H. and Thorburn, H. 1962. Serum antibodies to louping-ill virus. Scott. Med. J., 7, 353-355.

Williams, H., Thorburn, H. and Ziffo, G.S. 1963. Isolation of louping-ill virus from the red grouse. Nature, 200 , 193-194.

Wilson, D.R. 1945. Studies in louping-ill. I. Cultivation of louping-ill in vitro. II Pathogenic properties of culture virus. J. Comp. Path., 55, 250-267.

Wilson, D.R. and Gordon, W.S. 1948. Studies in louping-ill. IV Passive immunity. J. Comp. Path. 58, 210-226.

Zlotnik, I., Carter, G.B. and Grant, D.P. 1971. The persistence of louping-ill virus in immunosuppressed guinea-pigs. Br. J. Exp. Path., 52, 395-407.

Zlotnik, I., Keppie, J. and Grant, D.P. 1970. A method of testing the efficacy of louping-ill vaccines in sheep. Vet. Rec. 86, 659-660.

APPENDIX 3.1

PREPARATION AND POTENCY TESTING OF COMMERCIAL LOUPING-ILL VIRUS VACCINE

PREPARATION

Louping-ill virus is propagated in monolayer cultures of BHK-21 cells in BHK virus growth media and incubated as rolling cultures at 37°C. After 72 hours the supernatants are harvested in 4- or 8-litre volumes in 5- or 10-litre bottles. Samples are removed for infectivity titration using the plaque assay method. Formalin is added to the harvests to give a final concentration of 1/1000 and thoroughly mixed by shaking. After 24 hours incubation at 37°C samples are removed from the harvests and assayed for innocuity, sterility and residual formalin. The harvests are chilled at 4°C and cold methanol added (45ml methanol to each 100ml of harvest). The preparation is stirred continuously with a bar magnet for 24 hours at 4°C. The resulting precipitate is centrifuged at 1000g for 60 minutes at 4°C and resuspended to one tenth of the original volume in PBS pH 7.2 containing 0.5 per cent (v/v) phenol. Samples are removed from the 10-fold concentrate and tested for pre-blend potency. The bulk 10-fold concentrates are stored at -20°C until required.

POTENCY TESTING

Pre-blend potency test

Two-fold dilutions (1/2, 1/4 and 1/8) of the 10-fold concentrate samples are prepared in PBS + 0.5 per cent (v/v) phenol and separately emulsified with Bayol F and Falba oil adjuvant supplented with 0.5 per cent (v/v) phenol in a ratio 2 parts adjuvant to one part concentrate in a final volume of 15ml. Each preparation is then

potency tested according to the potency test specification. The response in sheep to these preparations determines to what extent the 10-fold concentrates can be diluted to prior to emulsification and preparation of the bulk vaccine.

Final potency test

The 10-fold concentrates are diluted and emulsified as and when required and a final potency test then carried out on a sample of the final containerized vaccine.

Prior to February 1979 the potency test specification stated that: "Not less than 6 healthy sheep devoid of louping-ill haemagglutination-inhibiting antibodies are each injected with 1.0ml of vaccine. Between 28 and 56 days after the injection the sera of at least 5 of the sheep must contain HAI antibodies at dilutions of 1/10 or greater ...". However batches of final vaccine have repeatedly failed to produce an immune response in accordance with this specification, and, to ensure protection following vaccination it was found necessary to recommend a double dose vaccination schedule and to revise the potency test specification as follows: "Not less than 6 healthy sheep devoid of louping-ill haemagglutination inhibiting antibodies are each injected with 1.0ml of vaccine. A second injection of 1.0ml of vaccine is given between 3 weeks and 6 months after the first. Between 15 and 56 days after the second injection the sera of at least 5 of the sheep must contain HAI antibodies at dilutions of 1/10 or greater ...".

The current recommendation for protection of sheep states that 2 1ml doses of vaccine should be administered and spaced not less than 3 weeks and not more than 6 months apart. The current recommendation

for the protection of cattle states that 2 2ml doses of vaccine should be administered and spaced not less than 3 weeks and not more than 6 months apart.

Appendix 3.2 The reciprocal HAI titres of Group I sheep following vaccination

Sheep No.	Response days post-vaccination				
	7	14	21	28	84
X153	160	640	80	80	640
X345	40	320	160	160	320
X475	<10	10	<10	<10	<10
X562	<10	<10	<10	<10	<10
X568	<10	80	40	<10	<10
X580	<10	<10	<10	<10	<10
X581	<10	<10	<10	<10	10
V693	<10	<10	40	40	80
V873	<10	<10	<10	<10	<10
V889	<10	<10	<10	<10	<10
Geometric mean	8.7	17	14	11	17

Appendix 3.3 The reciprocal HAI titres of Group II-VIII sheep immediately prior to and following revaccination

	Sheep No.	Response prior to revaccination	Response days post-revaccination 7	14	21	28	84
Group II	X543	<10	<10	10	<10	<10	20
	X351	<10	D				
	X342	<10	<10	<10	<10	<10	20
	X530	<10	<10	10	<10	<10	<10
	V860	<10	20	40	40	40	80
	X533	<10	<10	10	<10	<10	10
	X346	<10	40	20	20	<10	20
	X505	<10	640	640	640	160	80
	V877	<10	<10	20	20	10	40
	X471	<10	320	320	160	160	160
Geometric mean		5	20	20	22	15	29
Group III	X302	160	640	640	640	1280	640
	V692	20	640	320	160	320	640
	X335	160	1280	1280	640	1280	1280
	X515	40	80	80	80	80	160
	X432	<10	80	160	160	160	80
	X478	20	320	320	160	160	80
	X343	20	160	160	80	80	20
	X548	<10	<10	<10	<10	<10	<10
	X511	160	160	160	160	160	640
	V864	<10	20	10	<10	10	20
Geometric mean		26	138	129	91	120	120
Group IV	V896	10	160	80	1280	40	10
	X595	<10	20	80	160	80	640
	V888	20	1280	640	640	320	80
	V858	80	2560	1280	640	640	640
	X297	<10	80	80	80	40	10
	X457	<10	<10	<10	<10	<10	<10
	X300	80	640	640	1280	640	640
	V688	<10	160	160	80	40	<10
	X565	<10	40	20	40	<10	<10
	X563	<10	<10	<10	<10	<10	<10
Geometric mean		11	98	85	120	49	32
Group V	X467	<10	40	80	<10	40	<10
	V893	10	160	80	80	40	80
	V863	10	320	320	80	160	20
	X484	20	160	320	80	80	40
	X469	20	640	640	160	640	80
	V861	40	640	640	320	640	160
	X485	160	2560	2560	1280	2560	320
	X338	80	2560	10240	2560	2560	640
	V885	40	320	640	320	1280	1280
	X512	320	1280	10240	2560	2560	2560
Geometric mean		32	447	724	223	417	138

Appendix 3.3 cont'd

	Sheep No.	Response prior to revaccination	Response days post-revaccination 7	14	21	28	84
Group VI	X309	<10	20	<10	<10	<10	<10
	X464	1280	2560	2560	5120	5120	20480
	X463	320	1280	5120	5120	10240	10240
	X492	160	640	2560	2560	2560	10240
	X495	80	320	640	160	320	320
	X525	<10	40	40	20	20	80
	X536	<10	160	80	80	40	<10
	X547	<10	<10	<10	<10	<10	20
	X586	<10	40	20	20	<10	10
	V883	20	160	80	40	40	40
	Geometric mean	28	138	138	112	105	158
Group VII	X549	20	80	320	160	320	160
	X545	<10	80	80	40	160	<10
	X578	<10	160	80	80	320	10
	X157	<10	160	160	320	1280	40
	X579	640	320	640	640	2560	640
	X553	<10	<10	20	20	80	<10
	X490	<10	80	40	40	160	<10
	X577	10	80	40	40	640	20
	X359	20	160	160	80	160	<10
	X431	<10	320	160	160	640	40
	Geometric mean	11	98	105	91	363	21
Group VIII	X560	<10	1280	160	640	160	<10
	X155	10	40	80	320	40	10
	X436	10	160	160	640	320	20
	V695	D					
	V689	<10	160	80	320	160	20
	X524	<10	160	160	640	160	<10
	V869	10	20	40	160	160	40
	X466	20	640	640	2560	640	80
	X462	10	80	160	320	80	20
	X569	40	320	160	320	320	40
	Geometric mean	10	158	137	468	170	18

D: died through natural causes.

Appendix 3.4 The titres of virus (log_{10} pfu/ml) detected in sheep plasmas following inoculation with louping-ill virus

	Sheep No.	Days post-inoculation 1	2	3	4	5	6
Unvaccinated controls	X333	1.4	NT	4.3	3.7	3.6	N
	X477	N	N	N	0.7	N	N
	X497	0.4	2.0	2.0	2.3	N	N
	X501	N	N	N	N	N	N
	X520	1.4	2.0	2.6	3.2	1.6	N
	X538	2.0	2.9	4.9	4.9	3.3	N
	V875	1.1	2.7	4.6	6.9	5.4	2.0
	V876	0.7	0.4	1.5	2.8	3.6	N
	V894	1.6	2.5	3.7	3.4	N	N
	V897	1.0	2.4	3.9	5.8	2.4	N
Vaccinates: titre <10 at challenge	X475	N	N	N	N	N	N
	V889	1.4	2.1	4.2	4.5	4.0	N
	X342	N	N	N	1.1	N	N
	X543	N	N	N	2.3	1.1	N
	X548	1.0	1.2	2.8	1.7	1.7	N
	V864	N	1.5	2.9	3.8	N	N
	X565	N	N	3.1	1.4	N	N
	X563	1.1	1.3	2.6	N	N	N
	X586	N	1.9	3.6	3.0	3.4	N
	X490	N	1.0	0.9	N	N	N
	X553	N	N	N	N	N	N
Vaccinates: titre ≥10 at challenge	X467	1.4	1.9	2.6	2.4	0.9	N
	V893	N	N	N	N	N	N
	X309	0.4	N	0.9	N	N	N
	X155	N	N	N	N	N	N
	X462	N	N	N	N	N	N

N = virus not detected
NT = plasma not tested

Appendix 3.5 The reciprocal HAI titre (log_{10}) in sheep following inoculation with louping-ill virus

	Sheep No	Days post-inoculation 5	6	6*	7	8	8*	9	10	10*	11	12	12*	14	14*
Unvaccinated controls	X333	0.7	1.9	1.6	2.8	3.1	1.6	3.4	3.4	3.1	3.4	3.4	2.8	3.4	2.2
	X477	0.7	2.5	1.3	2.8	3.1	1.6	2.5	2.8	1.9	2.5	2.5	2.2	2.8	2.5
	X497	0.7	2.2	1.3	3.1	3.1	2.5	3.7	3.4	2.5	3.4	3.4	2.8	3.7	2.8
	X501	0.7	0.7	1.0	1.3	2.5	1.3	2.8	3.7	1.6	4.0	4.0	1.6	4.3	1.9
	X520	0.7	2.2	1.0	3.1	3.7	1.9	3.4	3.7	2.8	3.7	4.0	3.1	4.3	3.7
	X538	0.7	2.5	1.6	3.4	3.7	2.8	4.3	DEAD						
	V875	0.7	2.2	1.3	2.8	3.4	2.2	3.1	DEAD						
	V876	0.7	1.3	0.7	2.2	3.4	1.3	3.1	3.4	1.9	3.4	3.7	2.5	3.7	2.5
	V894	0.7	3.1	1.9	3.1	3.7	2.2	3.4	3.4	3.1	3.7	3.4	2.8	4.3	2.2
	V897	0.7	2.2	0.7	2.8	3.7	1.9	3.1	3.1	2.2	3.4	3.4	2.5	3.7	2.8
Vaccinates with titre <10 at challenge	X475	0.7	1.9	1.3	2.5	2.8	2.2	2.8	3.1	3.4	3.7	3.7	3.7	NT	
	V889	0.7	1.9	1.6	3.1	3.4	1.6	3.4	3.1	2.5	3.1	3.1	2.8		
	X342	0.7	2.5	1.9	3.4	3.4	1.9	3.7	3.7	3.7	3.7	4.0	4.0		
	X543	0.7	1.6	1.3	2.2	2.8	1.6	3.1	3.4	2.8	3.4	3.4	3.4		
	X548	0.7	2.2	1.6	3.4	4.0	3.1	4.0	4.0	3.7	4.3	3.7	4.0		
	V864	1.3	2.8	1.9	3.1	3.1	2.5	3.4	3.7	2.8	4.0	3.7	3.7		
	X565	1.9	3.1	1.3	3.1	3.1	1.9	3.7	3.7	3.1	3.7	4.0	3.4		
	X563	1.3	2.5	1.3	3.1	3.4	1.6	3.7	3.7	1.9	3.7	4.0	4.0		
	X586	0.7	2.8	1.6	3.4	3.7	2.2	4.3	4.0	3.4	4.0	4.0	4.0		
	X490	2.2	3.1	2.5	3.4	3.7	3.1	4.0	4.0	3.4	4.0	3.7	4.0		
	X553	2.2	2.8	2.2	2.8	3.4	2.5	3.1	3.4	3.1	3.1	3.4	3.1		
Vaccinates with titre ≥10 at challenge	X467	0.7	2.8	2.2	3.4	3.7	2.8	4.0	4.0	4.0	4.0	4.0	3.4	NT	
	V893	1.9	3.4	2.5	3.4	4.0	3.1	3.4	3.7	2.8	3.7	3.7	3.7		
	X309	0.7	2.5	1.9	3.1	3.1	2.2	3.7	2.8	2.8	3.1	2.8	3.1		
	X155	1.9	3.1	1.6	3.1	3.1	1.9	3.4	3.7	NT	3.1	3.4	3.4		
	X462	1.9	3.1	2.8	3.4	3.4	3.1	3.7	3.7	3.7	3.7	3.7	3.7		

*Sera heated 30 minutes at 64.5°C NT = not tested

Appendix 4.1 Infectivity titres (log_{10} pfu/ml) and reciprocal HA (log_{10}) titres of virus harvests propagated in BHK-21 cells in stationary and rolling tube culture

Hours post-infection	Stationary		Rolling	
	Infectivity	HA	Infectivity	HA
24	7.6	1.8	7.5	1.5
	7.7	1.8	7.8	1.5
	7.7		7.4	
48	7.3	2.1	7.1	2.1
	7.4	2.4	7.3	2.1
	7.5		7.2	
72	7.6	2.4	7.1	2.1
	7.7	2.4	7.2	1.8
	7.6		7.2	
96	8.0	2.4	7.3	2.4
	8.1	2.1	7.5	2.1
	8.0		7.5	
120	7.7	2.4	7.1	2.4
	7.8	2.1	7.4	2.4
	8.0		7.1	

Samples removed at each time interval were assayed for virus infectivity in triplicate and for HA activity in duplicate

Appendix 4.2 Infectivity titres (log_{10} pfu/ml) and reciprocal HA titres (log_{10}) of virus harvests propagated in BHK-21 tube cultures in BHK virus growth media containing either 10% LS or 5% LS

Hours post-infection	Serum content of virus growth media: 10%				5%			
	Stationary cultures		Rolling cultures		Stationary cultures		Rolling cultures	
	Infectivity	HA	Infectivity	HA	Infectivity	HA	Infectivity	HA
24	7.3	1.2	7.0	1.2	7.4	1.5	7.7	1.2
	7.2	1.2	7.0	1.2	7.9	1.5	7.7	1.2
48	8.1	2.4	7.6	2.1	7.0	1.5	7.3	1.5
	8.1	2.4	7.7	2.1	7.1	1.5	7.4	1.8
72	7.9	2.4	7.2	2.1	6.6	0.9	6.1	1.5
	7.9	2.4	7.2	2.1	6.6	0.9	6.5	1.5
96	7.8	2.7	6.9	2.4	4.2	0.3	6.2	1.2
	7.9	2.7	6.9	2.4	4.3	0.3	6.2	1.2
120	8.0	2.7	6.2	1.5	2.5	0.3	5.9	0.6
	7.9	2.7	6.0	1.5	2.4	0.3	5.9	1.2

Samples removed at each time interval were assayed for virus infectivity and HA activity in duplicate

Appendix 4.3 The infectivity titres (log_{10} pfu/ml) of virus harvests propagated in BHK-21 cells and BHK-21 (R8MF) cells in stationary and rolling tube cultures

Hours post-infection	BHK-21		BHK-21 (R8MF)	
	Stationary	Rolling	Stationary	Rolling
24	7.6	7.7	6.3	6.4
	7.5	7.7	6.1	6.3
	7.4	7.6	5.8	5.6
	7.5	7.7	6.0	5.6
48	7.4	7.6	6.0	6.2
	7.5	7.7	6.1	6.2
	7.4	7.3	6.0	5.2
	7.3	7.1	5.8	5.3
72	7.6	7.6	5.5	5.6
	7.4	7.3	5.5	5.7
	7.6	7.3	4.9	4.1
	7.6	7.5	5.0	4.1
96	8.0	7.5	6.6	5.0
	8.0	7.7	6.7	5.1
	7.9	7.3	5.5	3.0
	7.8	7.2	5.6	3.1
120	7.9	7.4	6.4	5.1
	7.9	7.5	6.5	5.0
	7.4	7.0	4.4	3.1
	7.5	7.2	4.3	3.0

Data collated from two separate experiments carried out under the same adsorption and growth conditions

In both experiments the samples removed at each time interval were assayed for virus infectivity in duplicate

Appendix 4.4 The reciprocal HA titres (log_{10}) of virus harvests propagated in BHK-21 cells and BHK-21 (R8MF) cells in rolling and stationary tube culture

Hours post-infection	BHK-21		BHK-21 (R8MF)	
	Stationary	Rolling	Stationary	Rolling
24	1.5	1.8	0.3	0.9
	1.2	1.8	0.3	0.9
	1.5	1.5	0.6	0.6
	1.2	1.5	0.6	0.6
48	2.4	2.1	1.2	1.2
	2.1	2.1	1.2	1.2
	2.1	2.1	1.2	0.9
	2.1	2.1	1.2	0.9
72	2.4	2.4	1.2	1.5
	2.4	2.4	1.2	1.5
	2.4	2.4	0.9	0.9
	2.4	2.4	0.9	0.9
96	2.7	2.1	1.5	0.9
	2.4	2.4	1.5	0.9
	2.7	2.4	1.8	0.6
	2.4	2.4	1.8	0.6
120	2.7	2.4	1.5	1.2
	2.7	2.4	1.5	1.2
	2.7	2.4	0.9	0.9
	2.7	2.4	0.9	0.9

Data collated from two separate experiments carried out under the same adsorption and growth conditions

In both experiments the samples removed at each time interval were assayed for HA activity in duplicate

Appendix 4.5 Infectivity titres (log_{10} pfu/ml) and reciprocal HA (log_{10}) titres of virus harvests propagated in Wheaton roller bottles for vaccine production 1978-79

A1 Harvest		A2 Harvest		A3 Harvest	
Infectivity	HA	Infectivity	HA	Infectivity	HA
8.2	2.4	7.7	2.4	8.2	2.1
8.4	2.7	7.9	2.4	8.4	2.1
8.2	2.7	8.0	2.4	8.5	2.1
8.5	2.7	7.7	2.4	8.7	2.4
7.7	2.4	8.1	2.4	8.6	2.4
8.0	2.4	7.8	2.4	8.4	2.1
7.7	2.4	7.9	2.4	8.4	2.4
8.2	2.7	±0.06		8.4	2.4
8.1	2.6			8.4	2.3
±0.10	±0.06			±0.05	±0.06

B1 Harvest		B2 Harvest		B3 Harvest	
Infectivity	HA	Infectivity	HA	Infectivity	HA
7.6	2.1	8.0	2.4	8.2	2.4
7.8	2.4	8.3	2.4	8.3	2.4
7.9	2.4	8.2	2.4	8.4	2.4
7.9	2.7	8.3	2.4	8.5	2.7
7.9	2.7	8.3	2.4	8.3	2.4
8.0	2.7	8.4	2.4	8.4	2.4
7.7	3.0	8.6	2.1	8.5	2.7
7.7	2.1	8.2	2.7	7.2	2.4
7.9	2.7	8.3	2.4	8.6	2.7
7.9	2.7	8.4	2.7	8.5	2.4
7.8	2.6	8.3	2.4	8.3	2.5
±0.03	±0.09	±0.04	±0.05	±0.12	±0.05

C1 Harvest		C2 Harvest		C3 Harvest	
Infectivity	HA	Infectivity	HA	Infectivity	HA
8.4	2.1	7.7	2.4	8.1	2.4
8.6	2.4	7.7	1.5	8.0	2.4
8.4	2.4	8.0	2.7	8.1	2.4
8.5	2.4	6.1	2.4	8.1	2.4
7.9	1.8	7.9	3.0	8.2	2.1
8.4	2.1	7.5	2.4	8.1	2.4
8.4	2.1	±0.34	±0.25	8.1	2.4
8.4	2.4			8.1	2.4
8.2	2.4			8.0	2.4
8.4	2.4			8.1	2.4
8.3	2.3			8.1	2.4
±0.06	±0.07			±0.01	±0.03

Appendix 4.5 (cont'd)

D1 Harvest		D2 Harvest		D3 Harvest	
Infectivity	HA	Infectivity	HA	Infectivity	HA
8.4	2.4	8.0	2.4	7.5	1.8
8.4	2.4	8.2	2.4	7.4	1.8
8.5	2.4	8.0	2.1	7.5	2.1
8.6	2.4	8.3	2.1	8.0	2.1
8.7	2.7	8.1	2.4	7.8	2.1
8.8	2.7	8.2	2.1	7.8	1.8
8.5	2.7	7.8	2.1	7.7	1.8
8.5	2.5	8.3	2.1	7.9	1.8
±0.05	±0.06	8.4	2.1	7.5	1.8
		8.1	1.8	7.7	1.8
		8.2	2.2	7.7	1.9
		±0.05	±0.06	±0.05	±0.04

E1 Harvest		E2 Harvest		E3 Harvest	
Infectivity	HA	Infectivity	HA	Infectivity	HA
8.3	2.4	8.4	2.1	8.3	2.1
8.3	2.7	8.5	2.4	8.3	2.1
8.3	2.7	8.3	2.1	8.4	2.1
8.4	2.7	8.5	2.4	8.6	2.1
8.4	2.7	8.5	2.4	8.3	2.4
8.3	2.7	8.6	2.4	8.6	2.4
8.3	2.7	8.6	2.4	8.3	2.4
8.5	2.7	8.7	2.4	8.5	2.4
8.3	2.7	8.7	2.4	8.3	2.4
8.5	2.7	8.5	2.1	8.4	2.1
8.3	2.7	8.5	2.3	8.4	2.3
±0.02	±0.03	±0.03	±0.04	±0.03	±0.05

F1 Harvest		F2 Harvest	
Infectivity	HA	Infectivity	HA
7.8	2.1	7.8	2.1
8.3	2.1	8.2	2.1
8.0	2.1	7.8	2.1
8.4	2.4	8.2	2.4
8.5	2.4	8.2	2.4
8.1	2.4	8.2	2.4
8.5	2.4	8.5	2.4
8.3	2.4	8.2	2.4
8.3	2.1	8.2	2.4
8.5	2.1	8.0	2.4
8.3	2.3	8.1	2.3
±0.07	±0.05	±0.06	±0.04

Replicate samples: The number of replicate samples withdrawn from each harvest was dependent on the total harvest volume.
Mean titre ± s.e.

Appendix 6.1 The mean reciprocal HAI response* (log_{10}) evoked by concentrated and unconcentrated vaccines prepared from harvest pools A-F

Days post-vaccination	Harvest Pool A					Harvest Pool B				
	Unconc.	10x	5x	2.5x	1.25x	Unconc.	10x	5x	2.5x	1.25x
7	0.8 ± 0.06	1.5 ± 0.25	0.7	0.7	0.7	0.9 ± 0.15	1.1 ± 0.37	0.7	0.7	0.7
14	1.1 ± 0.15	2.3 ± 0.29	1.4 ± 0.13	1.2 ± 0.24	1.1 ± 0.26	1.5 ± 0.27	1.6 ± 0.49	1.6 ± 0.10	1.7 ± 0.37	1.4 ± 0.60
21	1.6 ± 0.12	2.3 ± 0.19	1.7 ± 0.23	1.3 ± 0.29	1.3 ± 0.30	2.0 ± 0.17	1.7 ± 0.48	1.6 ± 0.15	1.5 ± 0.32	1.4 ± 0.27
28	1.6	2.6 ± 0.23	1.8 ± 0.23	1.5 ± 0.34	1.7 ± 0.23	2.0 ± 0.15	1.9 ± 0.50	1.7 ± 0.22	1.5 ± 0.35	1.5 ± 0.27

	Harvest Pool C					Harvest Pool D				
	Unconc.	10x	5x	2.5x	1.25x	Unconc.	10x	5x	2.5x	1.25x
7	1.0 ± 0.12	0.7	0.7	0.7	0.7	0.9 ± 0.10	1.2 ± 0.25	1.0 ± 0.27	0.7	0.7
14	2.0 ± 0.26	1.2 ± 0.17	1.1 ± 0.20	1.4 ± 0.26	1.2 ± 0.30	1.8 ± 0.25	1.7 ± 0.25	1.7 ± 0.29	1.5 ± 0.27	1.1 ± 0.13
21	2.4 ± 0.10	1.3 ± 0.08	1.2 ± 0.25	1.1 ± 0.19	1.3 ± 0.25	2.0 ± 0.23	1.7 ± 0.29	1.7 ± 0.28	1.5 ± 0.23	1.2 ± 0.19
28	2.3 ± 0.17	1.6 ± 0.08	1.5 ± 0.37	1.2 ± 0.21	1.5 ± 0.26	2.0 ± 0.23	1.8 ± 0.27	1.7 ± 0.24	1.4 ± 0.19	1.2 ± 0.18

*Mean titre ± s.e.

Appendix 6.1 cont'd.

Days post-vaccination	Harvest Pool E					Harvest Pool F				
	Unconc.	10x	5x	2.5x	1.25x	Unconc.	10x	5x	2.5x	1.25x
7	1.1 ±0.13	1.1 ±0.26	0.8 ±0.10	0.7	0.7	0.8 ±0.06	1.2 ±0.18	1.0 ±0.27	0.7	0.7
14	2.2 ±0.24	2.3 ±0.18	2.0 ±0.21	1.9 ±0.31	2.0 ±0.31	1.5 ±0.21	2.1 ±0.26	2.0 ±0.27	1.2 ±0.26	1.7 ±0.32
21	2.6 ±0.17	2.3 ±0.16	1.7 ±0.18	1.9 ±0.31	1.7 ±0.25	2.0 ±0.31	2.2 ±0.30	2.2 ±0.25	1.2 ±0.27	1.9 ±0.28
28*	2.7 ±0.06	2.6 ±0.21	1.8 ±0.27	2.0 ±0.28	1.9 ±0.28	1.8 ±0.27	1.9 ±0.26	1.8 ±0.17	1.2 ±0.27	1.6 ±0.34
42	3.3 ±0.06	3.7 ±0.22	2.9 ±0.24	2.6 ±0.16	2.8 ±0.30	2.6 ±0.15	2.9 ±0.28	2.8 ±0.10	2.1 ±0.34	2.8 ±0.25
56	3.8 ±0.13	3.5 ±0.13	2.8 ±0.22	3.1 ±0.18	3.1 ±0.30	3.0 ±0.13	2.4 ±0.31	2.4 ±0.15	2.0 ±0.33	2.6 ±0.23

*Sheep revaccinated with a further 1ml of vaccine

Appendix 8.1 The reciprocal HAI titres (log_{10}) in mice in response to louping-ill virus vaccines

Virus Antigen Concentration								
Methanol Precipitation			PEG Precipitation			Ultrafiltration		
1M	2M	3M	1P	2P	3P	1U	2U	3U
1.9	1.3	2.2	2.2	1.0	3.1	2.8	3.7	3.7
2.8	1.3	2.2	2.2	2.8	3.1	3.7	3.7	2.5
2.2	1.3	1.9	2.2	2.8	2.5	3.7	2.5	3.7
1.6	1.9	2.2	2.2	1.3	3.4	3.1	3.7	3.4
1.9 ± 0.13			2.4 ± 0.20			3.4 ± 0.14		

Mean $\pm$ s.e.

Antibody titres 21 days post-revaccination

Vaccines tested immediately following production

Appendix 8.2 The reciprocal HAI titres (log_{10}) in mice following vaccination with louping-ill virus vaccines previously stored at -20°C as emulsion or antigen concentrate

Weeks of Storage	Emulsion			Antigen Concentrate		
	3M	3P	3U	3M	3P	3U
4	1.3	1.3	2.5	1.0	1.9	2.5
	1.0	1.3	3.1	1.0	2.2	2.8
	0.7	1.0	3.1	1.0	1.6	3.1
	0.7	0.7	2.8	0.7	0.7	3.1
	0.9	1.1	2.9	0.9	1.6	2.9
Mean±s.e.	±0.14	±0.14	±0.14	±0.08	±0.32	±0.14
8	0.7	1.3	2.8	1.0	1.3	3.7
	1.0	1.6	3.7	0.7	1.6	3.1
	1.0	1.3	3.1	0.7	1.9	3.7
	0.7	1.0	3.4	0.7	2.2	2.8
	0.9	1.3	3.3	0.8	1.8	3.3
Mean±s.e.	±0.08	±0.12	±0.19	±0.08	±0.19	±0.23
12	1.0	0.7	2.2	0.7	1.3	3.1
	1.0	0.7	3.1	1.0	1.0	3.7
	1.0	0.7	2.5	0.7	1.0	3.7
	1.0	0.7	2.5	1.0	1.0	3.1
	1.0	0.7	2.6	0.9	1.1	3.4
Mean±s.e.			±0.19	±0.09	±0.08	±0.17
24	0.7	1.0	2.5	0.7	1.3	2.5
	0.7	0.7	2.2	0.7	1.3	3.1
	0.7	1.6	2.2	0.7	1.3	1.9
	0.7	1.6	NT	0.7	1.6	3.1
	0.7	1.2	2.3	0.7	1.4	2.7
Mean±s.e.		±0.23	±0.10		±0.08	±0.29

Antibody titre 21 days post-revaccination

M: concentrated by methanol precipitation

P: concentrated by PEG precipitation

U: concentrated by ultrafiltration

NT: not tested

Appendix 8.3 The reciprocal HAI titres (log_{10}) in mice following vaccination with louping-ill virus vaccines stored at 4°C as emulsion or antigen concentrate

Vaccine	Weeks of storage							
	4		8		12		24	
	Emulsion	Concentrate	Emulsion	Concentrate	Emulsion	Concentrate	Emulsion	Concentrate
1M	1.0	1.3	0.7	1.3	0.7	1.0	0.7	1.0
	0.7	1.0	0.7	1.6	0.7	0.7	0.7	1.0
	0.7	1.0	0.7	1.6	0.7	1.0	0.7	0.7
	0.7	0.7	0.7	1.3	0.7	1.0	0.7	0.7
2M	1.3	1.3	0.7	1.6	0.7	0.7	0.7	0.7
	1.0	1.3	1.6	1.6	1.0	1.0	0.7	1.0
	1.0	1.3	0.7	1.0	0.7	1.0	0.7	1.0
	0.7	0.7	0.7	1.9	0.7	1.0	0.7	0.7
3M	1.9	1.9	1.3	1.6	1.0	1.0	0.7	0.7
	1.6	1.3	1.3	2.2	1.0	0.7	0.7	1.0
	1.3	1.3	1.6	2.5	0.7	1.9	0.7	1.0
	NT	1.0	1.6	1.6	0.7	0.7	0.7	0.7
	1.1	1.2	1.0	1.7	0.8	1.0	0.7	0.9
Mean ± s.e.	±0.12	±0.09	±0.12	±0.12	±0.04	±0.09		±0.05

Antibody titres 21 days post-revaccination
M = concentrated by methanol precipitation
NT = not tested

Appendix 8.3 cont'd

Vaccine	Weeks of storage							
	4		8		12		24	
	Emulsion	Concentrate	Emulsion	Concentrate	Emulsion	Concentrate	Emulsion	Concentrate
1P	1.9	1.0	1.3	2.2	1.0	1.0	1.6	1.0
	1.3	0.7	0.7	1.3	1.0	1.3	1.6	1.0
	1.3	0.7	0.7	1.3	0.7	0.7	1.3	0.7
	1.0	0.7	0.7	0.7	1.0	1.9	1.0	0.7
2P	2.5	2.5	1.6	2.5	1.0	1.0	1.9	1.3
	1.6	2.2	1.3	2.5	1.0	2.2	1.3	1.3
	1.0	1.6	1.0	2.2	0.7	1.0	1.0	1.0
	0.7	1.3	2.2	1.9	1.9	0.7	1.3	0.7
3P	1.9	1.3	0.7	1.9	0.7	1.0	1.6	1.3
	1.0	1.0	0.7	0.7	1.0	1.0	1.0	1.0
	0.7	1.0	0.7	1.9	1.0	1.3	0.7	1.6
	0.7	1.0	1.0	0.7	1.6	1.0	1.0	NT
	1.3	1.3	1.1	1.7	1.1	1.2	1.3	1.1
Mean ± s.e.	±0.17	±0.17	±0.14	±0.20	±0.10	±0.13	±0.10	±0.09

Antibody titres 21 days post-revaccination
P = concentrated by PEG precipitation
NT = not tested

Appendix 8.3 cont'd

Vaccine	Weeks of storage: 4		8		12		24	
	Emulsion	Concentrate	Emulsion	Concentrate	Emulsion	Concentrate	Emulsion	Concentrate
1U	2.8	3.4	3.1	3.1	2.5	2.5	0.7	2.5
	3.7	3.1	2.8	2.2	3.4	2.5	2.2	1.0
	3.1	1.9	1.9	2.2	3.1	2.5	1.9	1.9
	3.1	2.8	2.8	3.1	2.8	2.2	2.5	2.2
2U	2.8	3.7	2.8	3.1	2.5	3.1	2.5	1.9
	3.4	3.7	3.1	2.8	2.8	2.5	3.7	0.7
	3.1	3.7	3.4	2.8	3.1	3.1	1.9	2.8
	2.5	3.1	2.8	2.8	3.7	2.8	NT	1.9
3U	3.4	3.7	2.5	3.1	3.4	3.1	2.5	2.5
	2.8	3.4	3.1	3.4	3.1	2.8	2.8	1.9
	2.2	3.1	2.2	3.1	3.1	2.2	2.8	2.8
	NT	3.1	2.5	2.5	1.9	2.5	1.6	1.9
Mean ± s.e.	3.0 ±0.13	3.2 ±0.15	2.8 ±0.12	2.9 ±0.11	3.0 ±0.14	2.7 ±0.09	2.3 ±0.23	2.0 ±0.19

Antibody titres 21 days post-revaccination
U = concentrated by ultrafiltration
NT = not tested

Appendix 8.4 The reciprocal HAI titres (log_{10}) in mice following vaccination with louping-ill virus vaccines stored at 'cool' as emulsion or antigen concentrate

Vaccine	Weeks of storage 1		2		4		8		12		24	
	E	C	E	C	E	C	E	C	E	C	E	C
1M	1.0	1.0	0.7	1.0	1.3	1.3	0.7	1.9	0.7	0.7	1.0	0.7
	NT	1.0	0.7	1.0	0.7	1.0	0.7	1.6	0.7	1.0	0.7	0.7
	NT	1.6	0.7	1.9	0.7	1.0	0.7	1.0	1.0	0.7	1.0	1.0
	NT	0.7	1.0	1.3	0.7	1.0	0.7	0.7	1.0	0.7	0.7	1.0
2M	0.7	0.7	1.0	1.6	1.0	1.6	0.7	0.7	1.0	1.0	1.0	0.7
	0.7	0.7	1.6	1.3	1.0	1.3	0.7	0.7	1.0	1.0	0.7	0.7
	0.7	0.7	1.3	1.0	0.7	1.0	0.7	0.7	0.7	1.0	1.0	0.7
	0.7	0.7	1.0	1.0	0.7	1.0	1.3	0.7	1.0	1.0	1.0	0.7
3M	0.7	1.0	0.7	1.0	1.9	1.6	1.0	1.0	0.7	0.7	0.7	0.7
	1.6	NT	1.0	1.0	1.6	1.3	0.7	1.9	1.0	1.0	0.7	0.7
	1.3	NT	1.3	1.0	0.7	1.0	0.7	1.0	0.7	0.7	0.7	0.7
	1.3	NT	1.3	1.0	0.7	1.0	0.7	1.6	NT	0.7	0.7	0.7
Mean ± s.e.	1.0 ±0.12	0.9 ±0.10	1.0 ±0.09	1.2 ±0.09	1.0 ±0.12	1.2 ±0.07	0.8 ±0.05	1.1 ±0.14	0.9 ±0.05	0.9 ±0.04	0.8 ±0.04	0.8 ±0.03

E = Emulsion
C = Concentrate

Antibody titres 21 days post-revaccination

M = concentrated by methanol precipitation

Appendix 8.4 cont'd

Vaccine	Weeks of storage											
	1		2		4		8		12		24	
	E	C	E	C	E	C	E	C	E	C	E	C
1P	0.7	1.3	0.7	1.6	1.0	1.6	0.7	1.3	1.0	1.0	1.0	0.7
	1.6	0.7	0.7	1.0	1.0	1.6	0.7	1.3	1.0	0.7	1.0	0.7
	0.7	0.7	1.0	1.3	1.0	1.6	0.7	1.9	1.0	0.7	1.6	0.7
	1.0	0.7	1.0	2.2	1.0	1.3	0.7	1.0	1.0	1.0	1.0	0.7
2P	0.7	2.2	0.7	1.9	1.0	1.6	0.7	2.5	0.7	1.0	1.0	1.0
	1.0	2.5	0.7	1.9	0.7	1.6	0.7	1.6	1.0	0.7	1.0	1.0
	2.5	2.5	1.9	1.3	0.7	1.3	0.7	1.3	1.0	1.0	1.0	0.7
	1.3	NT	1.3	3.1	0.7	1.3	0.7	1.6	1.0	0.7	1.0	1.0
3P	0.7	1.9	1.0	1.3	2.5	1.9	1.6	1.3	0.7	1.3	1.0	1.0
	0.7	2.5	0.7	1.9	1.3	1.9	1.3	0.7	1.0	1.0	0.7	0.7
	0.7	1.6	0.7	1.0	1.3	1.6	0.7	0.7	0.7	1.3	1.0	0.7
	0.7	1.6	0.7	1.6	1.0	1.0	0.7	0.7	1.0	1.0	0.7	1.3
	1.0	1.7	0.9	1.7	1.1	1.5	0.8	1.3	0.9	1.0	1.0	0.9
Mean ± s.e.	±0.16	±0.22	±0.16	±0.17	±0.14	±0.07	±0.08	±0.15	±0.04	±0.06	±0.06	±0.06

E = Emulsion
C = Concentrate

Antibody titres 21 days post-revaccination

P = concentrated by PEG precipitation

Appendix 8.4 cont'd

Vaccine	Weeks of storage											
	1		2		4		8		12		24	
	E	C	E	C	E	C	E	C	E	C	E	C
1U	2.8	1.3	3.7	3.7	3.7	2.8	3.4	3.7	1.0	1.6	0.7	2.8
	3.1	2.8	2.8	3.4	3.1	2.8	2.2	3.1	2.2	2.8	0.7	1.9
	3.7	3.7	2.2	3.4	3.4	2.5	1.6	3.1	0.7	2.8	1.9	0.7
	3.1	2.8	3.1	2.8	3.4	3.1	1.9	2.8	1.9	2.2	1.6	1.9
2U	1.9	2.8	2.8	2.2	3.1	2.5	2.2	2.5	2.5	3.1	1.9	1.6
	3.4	3.1	2.5	2.8	3.7	3.7	3.1	2.5	2.2	2.5	1.3	2.2
	3.1	2.8	2.5	2.2	3.1	3.1	1.3	2.2	1.0	2.8	1.0	2.2
	3.7	3.7	3.7	3.1	3.1	3.1	1.3	2.4	2.8	2.5	0.7	1.0
3U	2.8	2.8	2.5	2.2	2.5	3.7	1.9	2.8	2.2	2.8	3.4	1.9
	1.3	3.4	3.1	2.8	3.1	2.8	1.6	2.8	2.5	3.4	2.2	1.6
	2.8	3.7	2.8	2.5	2.2	1.9	2.5	3.1	1.9	3.4	1.9	2.8
	3.1	3.4	2.8	NT	1.9	3.1	2.2	2.5	2.5	3.7	2.8	1.3
Mean	2.9	3.0	2.9	2.8	3.0	2.9	2.1	2.8	2.0	2.8	1.7	1.8
$\pm$ s.e.	$\pm$0.20	$\pm$0.19	$\pm$0.13	$\pm$0.30	$\pm$0.16	$\pm$0.15	$\pm$0.19	$\pm$0.12	$\pm$0.20	$\pm$0.17	$\pm$0.24	$\pm$0.19

Temp °C						
Mean	10.0	12.0	12.2	14.6	16.4	17.6
Maximum	14.0	18.0	19.0	25.0	26.0	26.0
Minimum	8.0	8.0	8.0	8.0	8.0	8.0

E = Emulsion
C = Concentrate

Antibody titres 21 days post-revaccination

U = concentrated by ultrafiltration

Appendix 8.5 The reciprocal HAI titres (log_{10}) in mice following vaccination with louping-ill virus vaccines stored at 30°C as emulsion or antigen concentrate

Vaccine	Weeks of storage 1		2	
	Emulsion	Concentrate	Emulsion	Concentrate
1M	1.3	1.3	1.3	1.3
	0.7	1.0	1.0	1.3
	1.9	1.0	1.3	1.0
	1.3	1.0	1.3	1.0
2M	1.6	0.7	1.3	1.3
	0.7	0.7	1.3	1.3
	1.6	0.7	0.7	1.0
	1.9	1.6	1.0	1.3
3M	1.6	0.7	0.7	1.0
	1.6	1.0	1.0	1.0
	1.3	0.7	1.0	1.3
	1.3	1.9	1.0	1.9
	1.4	1.0	1.1	1.2
	±0.11	±0.11	±0.07	±0.07
1P	1.6	1.3	0.7	1.0
	1.6	0.7	1.0	1.0
	1.0	1.3	1.0	1.3
	0.7	0.7	1.0	1.0
2P	1.0	1.3	1.0	0.7
	0.7	0.7	1.0	0.7
	0.7	0.7	1.0	0.7
	0.7	0.7	1.0	0.7
3P	0.7	0.7	0.7	1.0
	0.7	1.6	1.0	1.0
	0.7	1.0	1.6	0.7
	0.7	1.0	1.3	0.7
	0.9	1.0	1.0	0.9
Mean ± s.e.	±0.10	±0.09	±0.07	±0.06

Antibody titres 21 days following revaccination

M = concentrated by methanol precipitation
P = concentrated by PEG precipitation

Appendix 8.5 cont'd

Vaccine	Weeks of storage			
	1		2	
	Emulsion	Concentrate	Emulsion	Concentrate
1U	0.7	2.5	1.9	2.5
	2.2	2.8	2.8	2.8
	3.4	2.8	2.2	1.9
	3.1	3.4	0.7	2.2
2U	2.2	2.8	1.9	3.1
	2.8	3.1	3.4	2.5
	1.6	3.1	1.9	2.8
	2.5	3.4	1.9	3.4
3U	3.4	3.1	1.9	2.2
	3.4	3.7	2.2	2.2
	2.5	3.1	2.5	2.2
	3.4	3.7	1.9	3.1
	2.6	3.1	2.1	2.6
Mean ± s.e.	±0.24	±0.10	±0.19	±0.13

Antibody titres 21 days following revaccination

U = concentrated by ultrafiltration